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THE UNIVERSITY OF ALBERTA

AN EXAMINATION OF NEOTENY IN *AMBYSTOMA TIGRINUM* OF TYRRELL'S LAKE, ALBERTA

by

GEORGE W. CORMIE

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "An Examination of Neoteny in *Ambystoma tigrinum* of Tyrrell's Lake, Alberta," submitted by George W. Cormie in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

A study of a neotenic population of the tiger salamander, *Ambystoma tigrinum*, of Tyrrell's Lake, Alberta, was undertaken between September 1969 and February 1971 to examine some aspects of its physiology and morphology.

The objectives of this study were threefold. The first was to determine why this particular population of *Ambystoma tigrinum* is neotenic and where, in the complicated hormonal system, the "normal" metamorphosis is being blocked. The second was to establish a numerical system for metamorphic stages based on changes in the external morphology during induced metamorphosis. The third objective was to determine whether injections of thyroxine would cause a change in the oxygen consumption of the neotenic *Ambystoma tigrinum*.

Metamorphosis was induced with L-thyroxine (T4) in order to create metamorphic stages for this population. Six stages, larval through adult, were selected, based on external characters such that the stage of induced metamorphosis could be determined without killing the animal. Each stage is described by a full lateral figure as well as a ventral and dorsal head figure. The stages were induced and therefore may not represent normal physiological or morphological changes associated with metamorphosis in non-neotenic populations.

Temperature, chemical and hormonal parameters were examined to determine at which point normal metamorphosis may have been interrupted thereby causing the larvae to be neotenic and paedogenetic. Increased temperature or addition of iodide (up to 40 µgm per liter) did not induce metamorphosis. Both Thyroxine and Thyroid Stimulating Hormone administered

by injection, caused the larvae to undergo metamorphosis. This would indicate that the blockage of metamorphosis was probably hypothalamic in nature and not due to thyroid dysfunction or lack of tissue sensitivity. The specific nature of this hypothalamic blockage could not be determined.

Oxygen consumption, measured one hour after injection, of T_4 treated *Ambystoma tigrinum* larvae was shown to be significantly lower than that of the sham injected controls. The oxygen consumption of larvae measured 24 hours after injection with T_4 was significantly lower than that of the larvae measured one hour after injection. It was not possible to measure the oxygen consumption of larvae throughout their induced metamorphosis although the trend of decreasing oxygen consumption did occur beyond the 24 hours post injection.

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INTRODUCTION

The biological phenomenon of amphibian metamorphosis was presumably well known to ancient peoples (Smith, 1969) and was often cited in the early texts of biology as one of nature's mysteries. It was not until the early part of this century that any attempt was made to discover the underlying mechanisms of this spectacular transformation.

In an early review by Allen (1929) the previous fifteen years of research were summarized in an attempt to draw some conclusions from the diverse experiments and results so far attained. Gundernatsh (1912, 1914) established the importance of the thyroid gland in metamorphosis when tadpoles of *Rana pipiens* exhibited precocious metamorphosis after feeding on ground bovine thyroid glands. With thyroidectomy experiments on young *Rana pipiens* tadpoles, Allen (1916) demonstrated that the removal of this gland prevented spontaneous metamorphosis, thus confirming its role in the metamorphic response. Once the importance of iodine in thyroid secretion, named thyroxine by Kendall (1915), was established (Marin and Rogoff, 1916), extensive research was carried out during the 1916 to 1929 period to determine if iodine played any other role in metamorphosis. The results of these experiments were largely inconclusive and contradictory and further investigation in this area was, for the most part, terminated.

It had long been known that there was a close relationship between the hypophysis and thyroid function (Allen, 1929), although the nature of the relationship was not fully understood. Experiments by Allen in 1929 on the hypothalamus and anterior pituitary of *Rana aurora drayton* demonstrated that hypophysectomy resulted in atrophy of the thyroid and

consequent failure of metamorphosis. This indicates that the thyroid, and therefore metamorphosis, are under the direct control of the hypothalamus-pituitary complex.

During the early period from the 1930's to the 1950's research was directed mostly to the effects of thyroxine on the metamorphic response, and very little toward hypothalamic-pituitary relationships. Thyroxine administered to thyroidectomized *Rana cantibrigensis* tadpoles was found to affect the rate of metamorphosis (increased dose—increased rate), but not the sequence of metamorphic events (Etkin, 1935). From work on the dose response to exogenous thyroxine and histology of the thyroid gland, it was hypothesized by Etkin (1935) that thyroxine levels in tadpoles gradually increased during metamorphosis to a high level at the climax period and then returned to pre-metamorphic levels. Since tissue sensitivity to thyroxine seemed to appear in all tissues at the same time (Etkin, 1950), there did not appear to be any explanation for the correct sequencing of metamorphic events during metamorphosis. With experiments on the effects of thyroxine-cholesterol pellet implants into various tissues in tadpoles, Kaltenbach (1953) confirmed the hypothesis that thyroxine was directly responsible for the metamorphic changes and that these changes were not a result of some other mechanism activated by metamorphosis. Study of thyroxine-like analogues was undertaken by Bruice, Winzler, Kharasch (1954) in an attempt to more fully understand the wide variety of metamorphosis-causing agents.

The complicated hormonal interrelationships between the hypothalamus, pituitary and thyroid were further studied in the period from 1960 to the present, thereby giving a more complete picture of the regulatory controls

of metamorphosis. Although the thyroid is capable of thyroxine production in early tadpoles (Flickinger, 1964), the low production of thyroid stimulating hormone (TSH) (Kollros, 1961) and the sensitive negative feedback of thyroxine on the pituitary TSH cells (Etkin, 1963) functions to keep thyroxine production low. Stimulation of the TSH cells in the anterior pituitary, as well as reduction of their high sensitivity to negative feedback of thyroxine by release factors from the hypothalamus, is necessary for increased TSH production and consequently increased thyroxine production (Etkin and Sussman, 1961; Etkin, 1963). Prolactin production by the anterior pituitary, unlike TSH production, is inhibited by hypothalamic release factors (Etkin and Gona, 1967). Therefore, in the absence of these release factors, prolactin is produced at a high rate. Prolactin is produced early in tadpoles and causes increased growth (Etkin and Gona, 1967) as well as having a negative feedback effect on the production of thyroxine from the thyroid (Gona, 1967; Gona and Etkin, 1970). Therefore, during the early larval stage, growth is stimulated by prolactin and metamorphosis is repressed because of low levels of thyroxine. The hypothalamus, which supplies the release factors to the pituitary via a portal system (rather than direct nervous connection) (Etkin and Sussman, 1961), is not sufficiently matured nor is the portal system developed enough to transport the hypothalamic factor, in order to increase TSH production, suppress prolactin production, and thus increase thyroxine levels sufficiently to cause metamorphosis (Etkin, 1963, 1965, 1966). Much of what Etkin and co-workers have hypothesized, in some instances, is not completely substantiated by their data. The component parts of the hormonal interactions in amphibian metamorphosis,

as described above, should be analyzed separately and therefore their hypothesis awaits further clarification.

As the hypothalamus is matured (which is somewhat dependent on low circulating levels of thyroxine [Etkin, Kikuyama and Rosenbluth, 1965]), production of TSH release factor and prolactin inhibitory factor seem to be positively stimulated by thyroxine (Etkin, 1966). As the result, TSH levels and thyroxine levels then rise and prolactin levels (and growth rate) drop, and the tadpole undergoes metamorphosis. At the metamorphic climax, and supposedly at a more mature stage of the hypothalamus, high circulating levels of thyroxine seem to reverse their effects on the hypothalamus and have a negative feedback effect to block the TSH release factor. This suppresses TSH secretion and returns the adult amphibian to premetamorphic circulating levels of thyroxine (Etkin, 1963).

A recent report by Van Oordt et al. (1972) based on detailed histological observation of the hypothalamus—pituitary—thyroid complex in *Xenopus laevis* contradicts certain aspects of the theories of Etkin and co-workers regarding the metamorphic process in amphibians. The part of Van Oordt's hypothesis regarding the role of prolactin in metamorphosis (which is also supported by the research of Norris and Platt, 1973, 1974; Norris, Jones and Criley, 1973) does not differ from that of Etkin (see text above). There is also no significant difference between the hypothesis of Van Oordt and that of Etkin with respect to the relationship between hypothalamic TRF and pituitary TSH on the production of thyroid hormones. The main point of disagreement lies in the feedback mechanism of thyroid hormones on the hypothalamus and the TRF cells. Etkin (1966, 1968) developed the theory that T4 caused maturation of both the hypothalamic

TRF cells and the portal system, as well as having a positive feedback effect on TRF production. This positive feedback increases the activity of the hypothalamic—pituitary—thyroid axis thereby creating an increase in circulating T4 levels and initiating metamorphosis. At metamorphic climax, high levels of circulating T4 cause the TRF cells to develop a negative sensitivity to this T4 and subsequently T4 levels fall (the normal adult condition). On the other hand, Van Oordt (1972) suggests that T4 stimulates differentiation of TRF cells, thus enlarging the TRF centre but exerts a negative feedback by lowering production of TRF by individual cells.

He therefore suggests that the larval hypothalamic—pituitary—thyroid complex is not substantially different from that of the adult. Thyroxine levels rise sharply during metamorphosis due to the morphogenic action of T4 on the hypothalamic TRF cells. When the differentiation of the TRF centre is completed (during metamorphic climax) T4 loses its morphogenic activity and the negative feedback remains. This causes a decrease in TSH production and therefore results in a lowering of T4 levels to the adult condition. Van Oordt's theory is substantiated by histological evidence and may prove to be more valid than that of Etkin's.

One is not necessarily justified in assuming that the proposed interaction of the hypothalamic—pituitary—thyroid complex applies to all species of amphibia. Rosenkildi (1972) suggests that there may be differences between species, "the regulation of thyroid activity in amphibia is characterized by a wide range of variation in the dependence of the thyroid upon the pars distalis of the pituitary and in the dependence of the pars distalis upon the central nervous system."

Besides the external and internal morphological changes and complex

hormonal interactions that accompany metamorphosis, there are a vast number of associated biochemical changes. Some of the research in this area has been on changes at the chromosomal and mitochondrial level (Cohen, 1970), while others have been on the basic enzyme systems (Frieden, 1968; Robinson, 1970). The various biochemical shifts are, of course, associated with the change from an aquatic life to that of a terrestrial one. Among the changes identified are: shifts from ammonotelism to ureotelism, increases in serum albumin and serum protein, changes in the molecular properties and biosynthesis of hemoglobin and alterations in digestive enzymes, respiration water balance and visual pigments (Frieden, 1961, 1967, 1968; Yamamoto, 1964; Yamamoto, Kanski and Frieden, 1966).

The species used in this study, *Ambystoma tigrinum*, is a common North American salamander and one of the few salamanders found in Alberta. *Ambystoma tigrinum* has a terrestrial, fossorial adult stage and an aquatic larval stage. Generally the adult (terrestrial) stage is reproductively mature, although in some instances the larvae may be paedogenetic (reproductively mature) (Dent, 1968). Fertilization is internal by way of spermatophores and eggs are laid in clusters on vegetation in slow moving streams, ponds, or lakes. The eggs hatch into the aquatic stage larvae which, after a period of growth (variable depending on temperature and availability of food), metamorphose into the terrestrial adults.

The *Ambystoma tigrinum* used in this work were collected from Tyrrell's Lake, a gully lake in southern Alberta, in the summers of 1970 and 1971. Other lakes and ponds in the area were reported by local farmers to contain similar "waterdogs" as they referred to them, but

most of these ponds proved to be inaccessible and difficult to seine. Tyrrell's Lake is heavily populated with *Ambystoma tigrinum* larvae measuring from a few centimeters to as much as 30 centimeters or more in length. Due to their large size and abundance it was evident that some or all of the population was neotenic (extended larval life) (Smith, 1969). There was also evidence to indicate that the larvae were sexually mature and thus paedogenetic (Dent, 1968). Reproductively mature *Ambystoma tigrinum* larvae could maintain a population of neotenic individuals in the absence of terrestrial adults, and therefore account for the large numbers of larvae and the lack of similar numbers of adults in the surrounding farmland.

The first objective of this study was to determine the reasons why this particular population of *Ambystoma tigrinum* is neotenic and at which point in the complicated hormonal system the normal metamorphosis is being blocked.

The second objective of this study was to establish a system of numbered metamorphic stages based on changes in the external morphology during the induced metamorphosis of the larval form of this particular population. Only two papers in the literature discuss staging of urodels and then only in broad general categories such as premetamorphosis, climax period and postmetamorphosis for four species: *Ambystoma punctatum*, *Triturus viridescens* (Grant, 1930) and *Ambystoma jeffersonianum*, *Ambystoma opacum* (Grant, 1931).

Oxygen consumption rates for larval, adult, and metamorphosing amphibians have been a highly disputed area of research from the 1930's to the present. Wills (1935), using small larval individuals of three species of urodels, including *Ambystoma tigrinum*, and two species of

anurans, found no sexual differences in oxygen consumption but did find an increased rate during metamorphosis. Helff (1927), using five species of *Ambystoma* including *A. tigrinum*, found great individual variation in oxygen consumption, although he did find the rates for the Mexican Axolotl (*Ambystoma tigrinum mexicanum*) were similar to those for *Ambystoma tigrinum*. Using anurans, Fletcher and Myant (1959) found a decrease in oxygen consumption during induced metamorphosis while Lewis and Frieden (1959) found the reverse during induced metamorphosis. Funkhouser and Mills (1969) found that oxygen consumption decreased or remained the same during induced metamorphosis in *Phyllobates* and decreased during spontaneous metamorphosis. Due to the wide variety of amphibians used and the varying techniques, it is not clearly established how oxygen consumption changes, if indeed it does change, during either induced or spontaneous metamorphosis.

The third objective of this study was to determine if injections of thyroxine would cause a change in the oxygen consumption of neotenic larval *Ambystoma tigrinum*.

MATERIALS AND METHODS

Background Information on *Ambystoma tigrinum*

A. Location of Animal Supply and Sampling Procedure

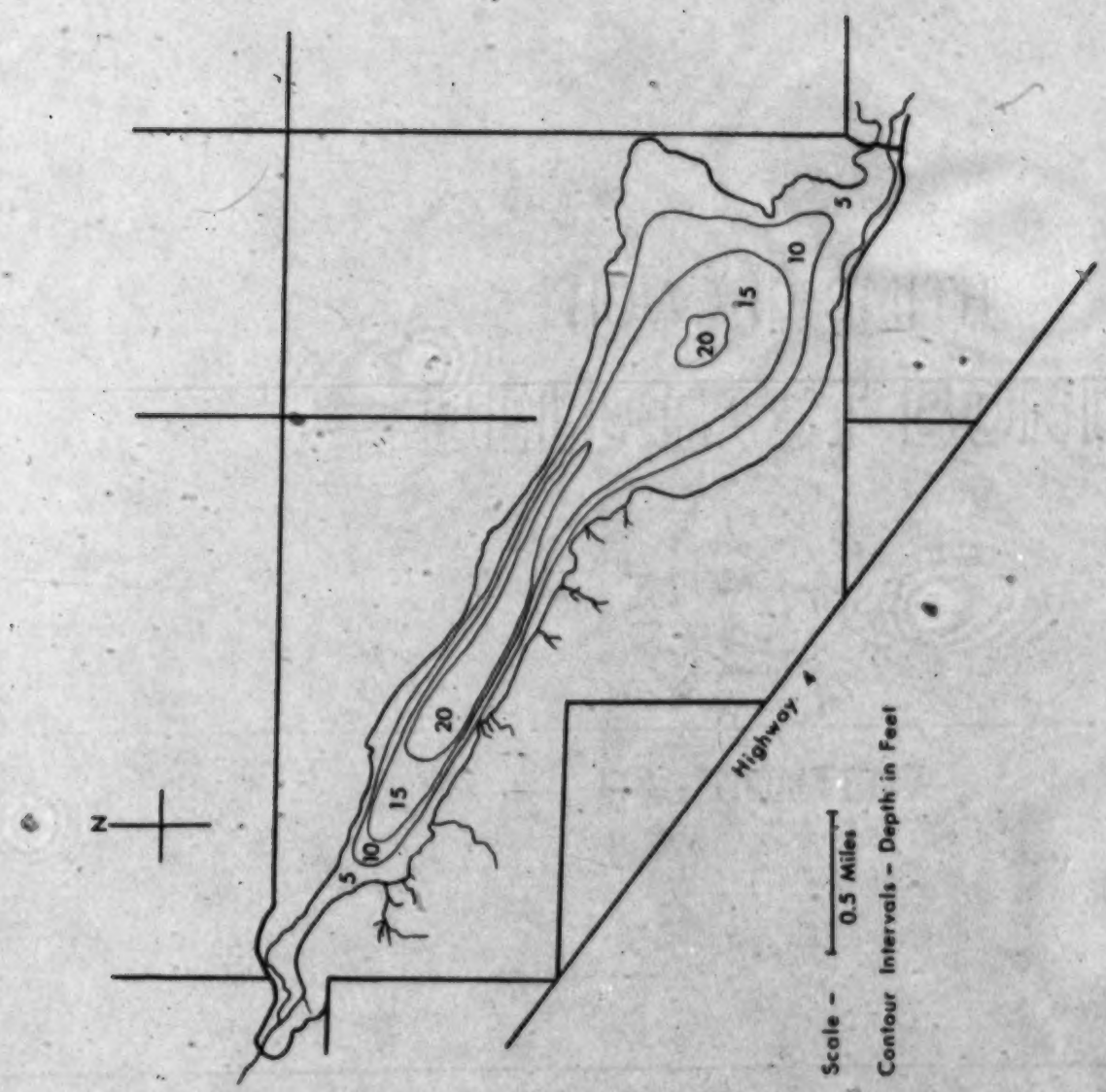
The larval *Ambystoma tigrinum* used in these experiments were collected from Tyrrell's Lake, a small, relatively shallow prairie lake, located 35 miles southeast of Lethbridge, Alberta, on highway 4 (Fig. 1). The area of the lake is approximately 980 acres (Lake Survey Report, see Appendix II), with masses of floating filamentous algae, 2 to 3 feet thick, extending as much as 20 feet from the shoreline along much of the leeward side of the lake. The lake is high in total dissolved solids (2822 ppm, Lake Survey Report; 5433 ppm, water analysis 1973, see Appendix III), most of which is sulfates. It is also somewhat alkaline (pH 8.2, Lake Survey Report; 9.1, water analysis 1973), the nature of the alkalinity being bicarbonates of calcium and magnesium.

The collecting site was located at the northwest end of the lake, immediately south of the narrows. The depth of the water in this area was no greater than 10 feet and the shoreline was accessible by truck. Other sites could also provide sufficient samples but due to their inaccessibility all sample collecting was restricted to the above mentioned site.

Collecting was carried out between dusk and midnight since the larval *Ambystoma tigrinum* exhibited vertical migration and seining during daylight hours was unsuccessful.

The most efficient method of seining was arrived at only after some

Fig. 1. Tyrrell's Lake, Sections 17, 18 and 19, Twp. 5, Rge. 17, W 4th, and Section 24, Twp. 5, Rge. 18, W 4th, approximately 35 miles southeast of Lethbridge.


5
10
15
20
20
15
10
5
N
Highway 4
Scale - 0.5 Miles
Contour Intervals - Depth in Feet

experimentation and was carried out in the following manner. A one hundred foot rope was attached to a triangular rope harness at each end of a six foot by forty foot seine. The seine was folded into an aluminum boat and one of the end ropes was secured on shore. The boat was then rowed out in a line perpendicular to the shoreline, the full length of the secured rope (100 feet). After the seine was let out parallel to the shoreline, the boat was rowed back to shore releasing the other 100 feet of rope. This procedure allowed the seine to be pulled from shore giving a six by forty by one hundred foot sweep of the lake. This sampling method was repeated until a sufficient sample of *Ambystoma tigrinum* larva was collected. Animals were collected for research in May and August of 1970 although the most profitable collecting was done in October of 1970.

B. Storage and Maintenance of Larval *Ambystoma tigrinum* in the Laboratory

Prior to the beginning of the study, various methods of storage of the larval *Ambystoma* were tried, to determine which would be the most suitable for the long term maintenance of these animals during the winter months. Freshly caught larvae were placed in holding tanks at temperatures approximating that of the lake (15°C to 20°C) and an attempt was made to feed them once they arrived in the laboratory. Ground pork liver, beef liver, beef heart or ground beef was provided at various times during the day. The larvae appeared to have no preference for any of these foods, and ate little or nothing whether presented during the light or dark periods. The problem of bacterial and fungal infection of the

larvae was evident with temperatures above 10°C and feeding only enhanced this because of the particles of food and feces in the holding tanks. Once the skin infection (which was not identified) appeared on larvae in a holding tank above 10°C, 80 to 100 per cent mortality occurred in two to three weeks. Animals held at temperatures of 4 to 5°C showed no signs of this infection and remained in apparently good physical condition for periods of up to eight months. At this temperature no feeding was required, mortality was low, and crowding created no additional health problems.

Subsequently all the larval *Ambystoma* caught for this study were placed as soon as possible in large holding tanks (3075 liters) of chilled water (4 to 5°C) in the Biological Sciences Aquatic Facilities. The water temperature was maintained by a portable cooling unit.

C. Experimental Design

Staging of Metamorphosis

Forty *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) and coloration were selected from the holding tank and placed in three 291 liter flow-through tanks at 25°C for two weeks. The larvae were removed from the 4 to 5°C water and raised to the experimental temperature (25°C) over a period of two days. As there was no method by which the *Ambystoma tigrinum* larvae could be aged, *size* was the parameter for selection of experimental individuals. All the larvae were then given single injections of 1 µgm L-thyroxine (T_4) per gram wet weight of animal. L-thyroxine (sodium salt) was dissolved in 50% aqueous solution of distilled water and propylene glycol in a concentration of 1 µgm per 1 µl.

It was administered by means of a 250 µl Hamilton syringe (in 2 µl divisions) with a 27 guage ½ inch disposable needle. Dose in all injections in the study was weight dependent (µgm T_4 per gram wet weight of animal). Commencing on the day of the injection a sample of three larvae (one from each tank) was taken every third day and placed immediately in a solution of 10% aqueous formalin. Any dead larvae were also taken from the tanks as soon after death as possible and fixed in the same manner. Although there was some mortality from fungal infections, samples were collected for the 30 days. In this manner a range of animals was obtained from the larval through the adult stage. After the animals were thoroughly fixed (usually one week) in 10% formalin they were transferred to 50% aqueous propyl alcohol so they could be more easily handled.

Changes in external morphology were examined in the sequence of sampled animals and six arbitrary stages were set up, with the true larva as the first stage and the adult as the sixth stage, with four intermediate stages of development. Drawings of the six stages (see Results) included a full lateral plus the dorsal and ventral head regions. A table of the important morphological characteristics of each stage was also compiled (Appendix I) and, together with the drawings, facilitated the separation of developmental stages. The morphological parameters chosen were not necessarily indicators of important physiological changes, but rather those which could be most easily identified by visual examination. External morphological criteria were used rather than internal ones so that killing the animal would not be necessary to identify its stage of development.

Thyroxine Experiments

Two experiments were conducted to determine the effects of L-thyroxine (T_4) on larval *Ambystoma tigrinum* and their metamorphosis. L-thyroxine was administered as described above.

a) Experiment 1. Fifty *Ambystoma tigrinum* larvae of similar size (10 to 28 cm) were randomly selected from the holding tank and 25 placed in each 291 liter flow-through tank and brought up to temperatures of 15 and 20°C respectively. The larvae were acclimated at these temperatures for two weeks. The larvae at each temperature were then divided into five groups of five larvae each. The following injection was then given to each larval *Ambystoma tigrinum* in the corresponding tank at each temperature: tank one, 1 µl per gram wet weight of 50% aqueous propylene glycol; tank two, 4 µgm T_4 per gram wet weight; tank three, 1 µgm T_4 per gram wet weight; tank four, 0.25 µgm T_4 per gram wet weight and tank five 0.0625 µgm T_4 per gram wet weight. A positive or negative metamorphic response (no change from stage I) was then recorded as well as any daily weight change.

b) Experiment 2. The second thyroxine experiment was run in conjunction with temperature experiment 2 and procedures are included under that heading.

Temperature Experiments

Most of the experiments were conducted at two or more temperatures to observe the influence of this parameter on the factor being tested. Two experiments were specifically designed to test the effects of temperature on the metamorphosis of the larva. All temperature changes from holding temperature to test temperature were accomplished over a

two day period.

a) Experiment 1. Forty *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) were selected from the holding tank and ten placed in each 291 liter flow-through tank and the temperature brought up to 10, 15, 20 or 25°C, respectively. Temperatures were maintained by means of mixing valves on each tank. The larvae were observed for 21 days for any signs of metamorphosis and then killed.

b) Experiment 2. Ninety-six *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) were randomly selected from the holding tank as controls. Twenty-four larvae were placed in each 291 liter flow-through tank and the temperatures brought up to 10, 15, 20 or 25°C, respectively. Another 96 experimental animals were also randomly selected from the holding tank and treated in a similar manner. All 192 larvae were then acclimated at their respective temperatures for two weeks. Following this, the experimental larvae were given single injections of T_4 at a dose of 1 µgm T_4 per gram wet weight of animal (see above). Each tank was checked daily to determine which metamorphic stage the larvae in the tank had reached. Records were kept as to the first and last day the larvae in any one tank reached a particular metamorphic stage, as well as the mortality in the tank. Due to the problem of infections it was impossible to physically tag any individual, although individual markings permitted reasonable identification.

Iodide Experiments

One experiment was conducted to determine the effects, if any, of iodide on triggering the onset of metamorphosis in *Ambystoma tigrinum* larvae.

One hundred and twenty larvae of similar size (10 to 28 cm) were selected randomly from the holding tank and 40 placed in each 291 liter flow-through tank and brought up to the temperatures of 15, 20 and 25°C respectively. The larvae were acclimated at these temperatures for two weeks. The larvae at each temperature were then further divided into four groups of ten and placed in separate 103 liter standing-water tanks at their respective acclimation temperatures. Because the tanks could not be temperature controlled with mixing valves, the temperatures were maintained with portable cooling and heating units. The corresponding tanks at each temperature were given the following treatments: tank one, 40 µgm potassium iodide per liter of water; tank two, 20 µgm potassium iodide per liter of water; tank three, 5 µgm potassium iodide per liter of water and tank four were the controls. The larvae were then observed for three weeks for any signs of metamorphosis.

Thyroid Stimulating Hormone Experiments

Three experiments were conducted to determine the response of the *Ambystoma tigrinum* larvae to thyroid stimulating hormone (TSH). Bovine thyroid stimulating hormone (Nutritional Biochemical Corporation) was dissolved in amphibian saline at a concentration of 1 International Unit per 100 µl. Injections were done subcutaneously with a glass 250 µl Hamilton syringe with a disposable 27 guage ½ inch needle.

a) Experiment 1. Forty *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) were randomly selected from the holding tank and 20 placed in each of 291 liter flow-through tanks and brought up to temperatures of 20 and 25°C respectively. The larvae were acclimated at these temperatures for two weeks. The larvae at each temperature were then further

separated into four groups of five and placed in separate 291 liter standing-water tanks at their acclimation temperatures. The temperatures were maintained with portable cooling and heating units. The following injection was then given to each larva in the corresponding tank at each temperature: tank one, 1 International Unit (I.U.) of TSH per animal; tank two, 0.5 I.U. TSH per animal; tank three, 0.25 I.U. TSH per animal and tank four, 100 µl of amphibian saline per animal. The animals were then observed for two weeks for any signs of metamorphosis.

b) Experiment 2. Thirty *Ambystoma tigrinum* larvae of similar size were randomly selected from the holding tank and 15 placed in each of 291 liter flow-through tanks and raised to the temperatures of 20 and 25°C respectively. The larvae were acclimated at these temperatures for two weeks. The larvae at each temperature were then further separated into three groups of five and placed in separate 291 liter standing-water tanks at their acclimation temperatures. Temperatures were maintained as described above. The animals in the corresponding tanks at each temperature were then given the following treatments: tank one, each larva was given a single injection of 1 I.U. TSH and 40 µgm potassium iodide per liter of water was added; tank two, each larva was given a single injection of 1 I.U. TSH and tank three, each larva was given a single injection of 100 µl of amphibian saline. The animals were then observed for two weeks for any signs of metamorphosis.

c) Experiment 3. The format of this experiment was identical in all respects to that of Experiment 2 except that multiple injections were given to the larvae. Injections were given once daily at the same dose as Experiment 2, for six consecutive days. The animals were also observed

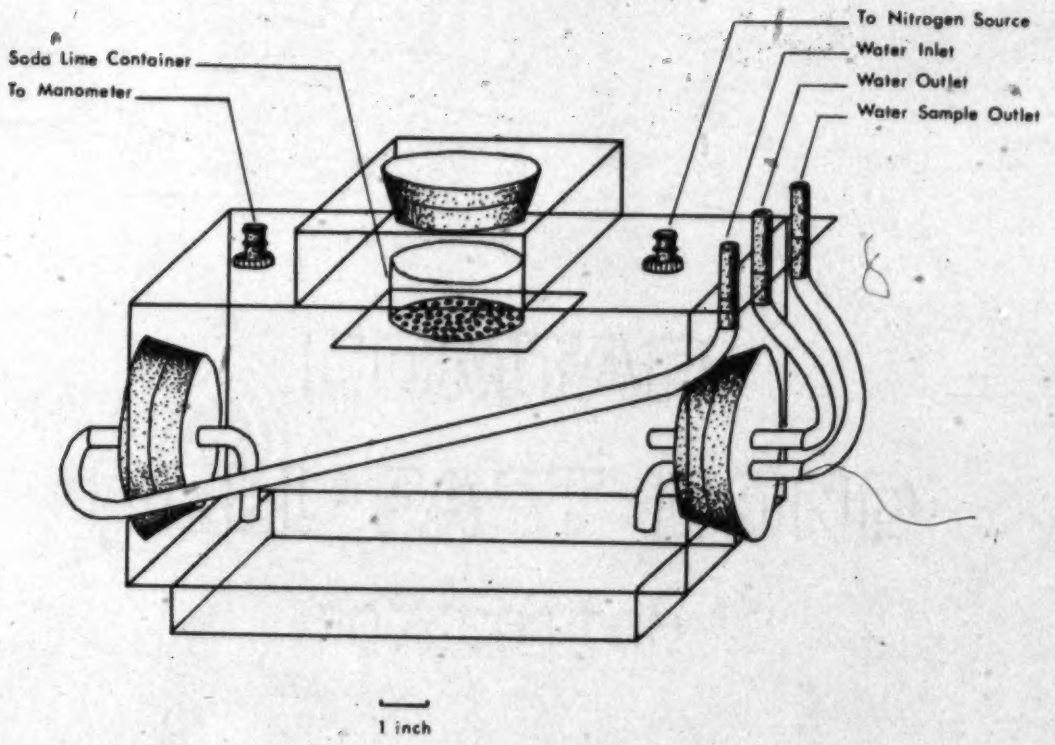
for two weeks, following the last injection, for any sign of metamorphosis.

Oxygen Consumption Experiments

A. Experimental Apparatus. For the whole animal respiration experiments, a respiratory chamber was constructed from ¼ inch plexiglass in such a manner as to contain half water (1735 to 1743 cc) and half air (Fig. 2, Plate 3). This would allow the *Ambystoma tigrinum* larva to use gill, skin or lung respiration, since all are functional in the larva. A container of soda lime (indicator grade) was suspended in the upper portion of the chamber to absorb the carbon dioxide produced by the larva during the experiment. Only the stopper above the soda lime and the stopper at the water inlet end were removable. The other stopper was cemented in place with silicon cement, in order that the water outlet tube would remain constant from experiment to experiment. All tubing (tygon), water inlet, water outlet and water sampling tube, were fixed to the top of the chamber at the water outlet end to facilitate chamber hookup at the beginning of each experiment. Four such chambers were built since only four could be placed in the constant temperature bath at any one time.

The water bath was constructed from a 26 inch by 50 inch by 12 inch deep insulated fiberglass tank. A 0.5 inch copper pipe cooling coil circled the inner perimeter of the tank four times, thus leaving the center of the tank free for the respiration chambers. This cooling coil was connected to the inlet and outlet of a modified Fisher Isotherm Bath with tygon tubing. The temperature control unit on the Fisher Isotherm Bath was replaced by a YSI temperature control unit. A thermister probe in the water bath was connected to the temperature control unit on the

Fig. 2. The plexiglass chamber used for the measurements of oxygen consumption in the larval *Ambystoma tigrinum*.


To Nitrogen Source
Water Inlet
Water Outlet
Water Sample Outlet
Soda Lime Container
To Manometer
1 inch

Fisher Isotherm Bath, which in turn controlled the temperature of the circulating fluid in the coils of the constant temperature bath. This apparatus maintained the temperature of the bath to within ± 1°C of the set temperature.

A nitrogen cylinder and two stage regulators provided a source of nitrogen for equalizing the animal chamber pressure during the experiment. The respiration chambers were connected to the nitrogen source as shown in Figure 3. The chambers in turn were connected to the manometer set-up and compensation chamber as shown in Figure 4.

At the start of each experiment the larvae were blotted, weighed, and placed in numbered chambers through the water inlet end; then the stopper was inserted and secured (Plate 1). Fresh soda lime was placed in the plastic mesh dish in each chamber and the stopper above it secured. The chambers were then immersed in the large constant temperature bath and a lead ballast was placed in a shelf attached to the underside of each chamber to compensate for the chamber buoyancy. The tube leading from the manometer set-up and the one leading from a 10 ml syringe and nitrogen source were then connected to their respective brass nipples on the top of each respiration chamber. The open ends of the manometers were connected to a compensation chamber in the waterbath to correct for small changes in pressure or temperature (Fig. 4). The water inlets of each chamber were then connected to a water reservoir (gravity flow) and the water outlets were connected to a drain. The water sampling outlet tube (fitted with a 3-way metal syringe fitting) was attached to a 10 ml glass syringe with a glass tip (water sampling syringe).

Fig. 3. The diagrammatic organization of the nitrogen source and syringes used in the re-equilibration of the chamber pressures during an experiment.

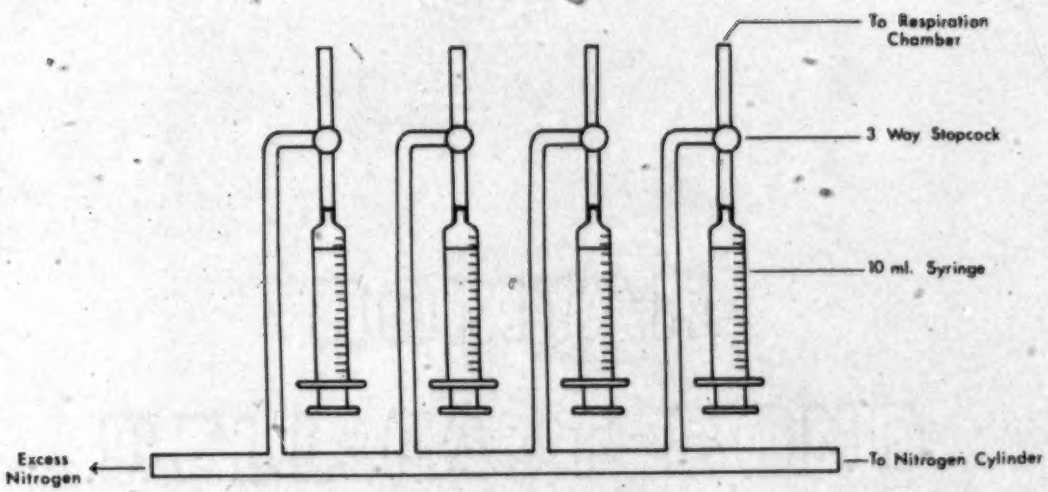
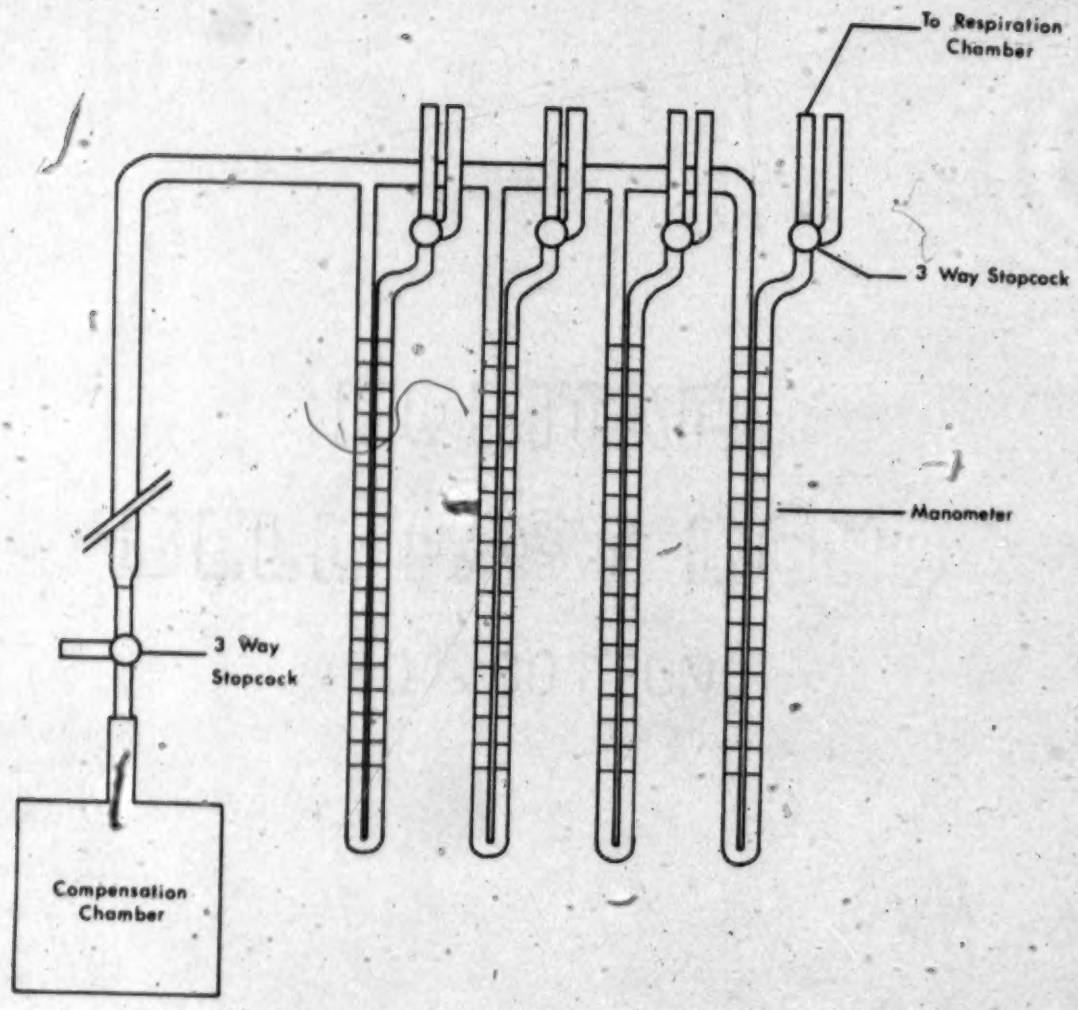
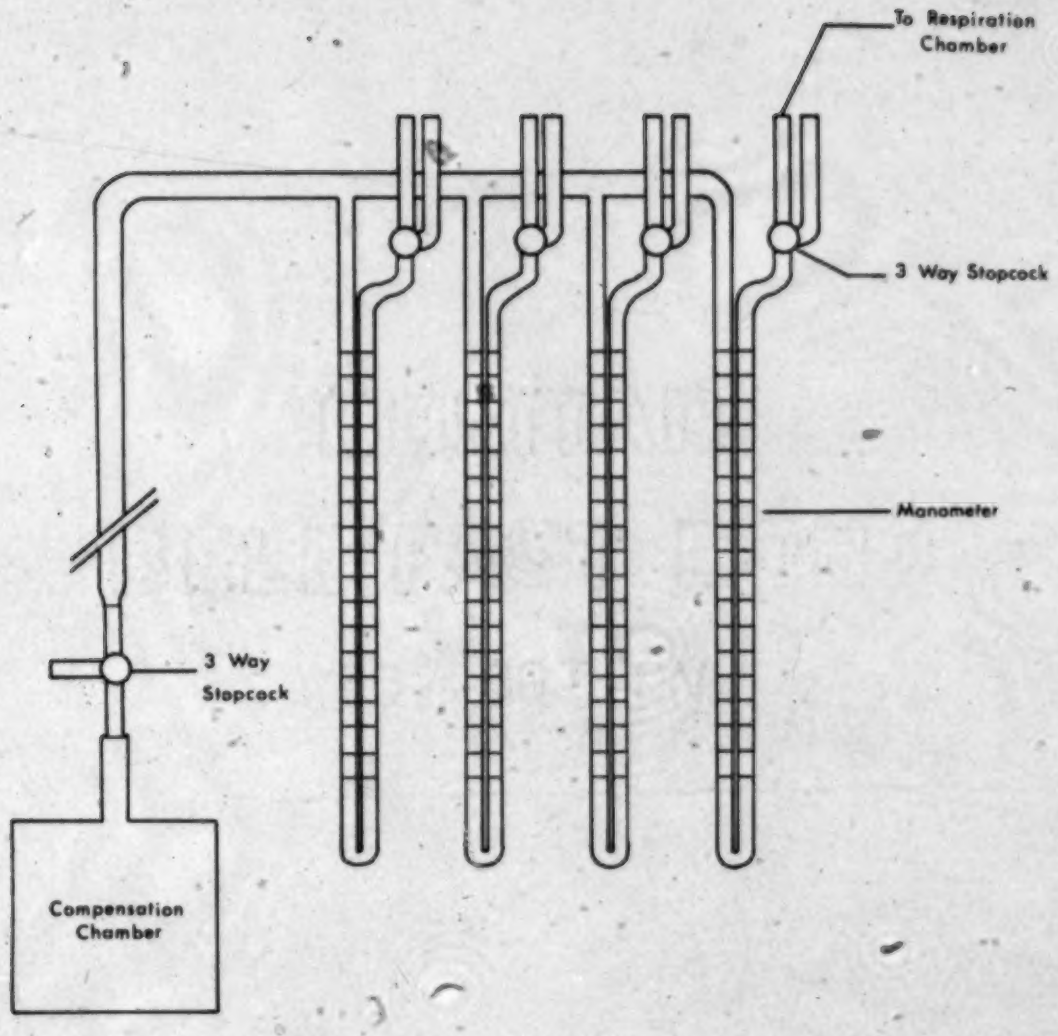

To Respiration Chamber
3 Way Stopcock
10 ml. Syringe
Excess Nitrogen
To Nitrogen Cylinder

Fig. 4. The diagrammatic organization of the manometers and compensation chamber in relation to the respiration chambers.


To Respiration Chamber
3 Way Stopcock
Manometer
3 Way Stopcock
Compensation Chamber


To Respiration Chamber
3 Way Stopcock
Manometer
3 Way Stopcock
Compensation Chamber

When all the tubing was properly connected, the manometer tubing and nitrogen source tubing (leading from their respective brass nipples on the top of the respiration chambers) and water sampling syringe were closed off by way of 3-way glass stopcocks and the water inlet tube was opened (pinch clamp). When the water had reached the correct level within the chambers (up to the level of the water outlet tubing), it was allowed to flow for five additional minutes then the water inlets were clamped off. The nitrogen source tubing was then opened to the chamber and the chamber was flushed with oxygen in the following manner: the tube attached to the nitrogen source was removed and connected to an oxygen cylinder and two stage regulators. The chamber was then gassed for 0.5 minutes at approximately 2 lbs/sq in (the water outlet tube allowed the excess gas to escape); after the 0.5 minutes the oxygen was turned off and the tube was then reconnected to the nitrogen source. The chambers were then left to equilibrate for 1 hour. After the equilibration time, three 10 ml water samples were taken from the water sampling tube in each chamber using the 3-way syringe stopcock and 10 ml glass syringes. The nitrogen source tubing was then closed off from the chambers (3-way glass stopcock), the manometers were opened to the chambers (3-way glass stopcock), the compensation chamber was connected and the water outlet tube was closed off (pinch clamp), thus forming a closed respirometer.

During the five hours that these respirometers were in operation, air pressure within them changed as the oxygen was directly consumed by the animal or absorbed into the water. The amount of oxygen removed from the air space above the water in each chamber (either consumed by the

animal or by absorption into the water) was recorded on the manometer and quantitatively measured by injecting nitrogen to reestablish the original level. The total amount of nitrogen injected was recorded. At the termination of the experiment three more water samples were taken as before and the animals were replaced in their holding tanks. The increase or decrease of oxygen in the water was determined by the Winkler method (Burt, 1962) on the water samples taken at the beginning and the end of each experimental period.

The data for each chamber was recorded on a data sheet run through an A.P.L. terminal computer to calculate the oxygen consumption of each larva (Appendix VI).

B. Oxygen Consumption Experiments.

a) Experiment 1. Forty *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) were selected from the holding tank and placed in a 29l liter flow-through tank and brought up to 15°C. The larvae were acclimated at this temperature for a minimum of two weeks. The following treatments were given to the larvae:

Treatment 1. Four *Ambystoma tigrinum* larvae at a time were each given an injection of 1 µl of 50% propylene glycol (aqueous solution) per gram wet weight of animal, one hour prior to the measurement of oxygen consumption at 15°C.

Treatment 2. Twenty-four hours after treatment 1, the same *Ambystoma tigrinum* larvae were each given a single injection of T_4 (1 µgm T_4 per gram wet weight, at a concentration of 1 µgm T_4 per 1 µl of 50% propylene glycol) one hour prior to the measurement of oxygen consumption at 15°C.

The oxygen consumption of four larvae was measured during each experimental period, using the procedure given in the previous section for experimental apparatus, until all 40 larvae had been studied both as sham-injected (control) or T_4-injected. Except for the 20 larvae used subsequently in Experiment 2, all injected (T_4) *Ambystoma tigrinum* larvae were killed after the oxygen consumption measurement to avoid mixing them with the uninjected larvae.

b) Experiment 2. The oxygen consumption of 20 of the larvae from Treatment 2 was also measured at twenty-four hours post T_4 injection. These larva were then killed to avoid mixing them with the uninjected larvae.

c) Experiment 3. Eight *Ambystoma tigrinum* larvae of similar size (18 to 28 cm) were selected from the holding tank and placed in a 291 liter flow-through tank and brought up to 15°C. The larvae were acclimated at this temperature for two weeks. Each larva was marked with a numbered metal fish tag on the tail for individual identification. Four of the larvae were given sham injections (1 µl of 50% propylene glycol per gram wet weight), and four were given injections of T_4 (1 µgm T_4 per gram wet weight—dose as above in Experiment 1). The larvae were then divided into two groups of four larvae each, in such a manner that two members of each treatment were included (two sham and two T_4) during the measurement of oxygen consumption. The oxygen consumption of each salamander in the two groups was measured daily for ten days.

Wet and Dry Weight Determinations

Twelve *Ambystoma tigrinum* larvae of greatly varying weights and lengths were selected from the holding tank and weighed. They were killed,

placed on large preweighed filter papers and cut open. The remains on their respective filter papers were then placed on racks in an oven at 100°C. The papers were kept in the oven until their weights remained constant. The dry filter papers with animal remains were then weighed and their final dry weights calculated.

RESULTS

Larval Development

The *Ambystoma tigrinum* larvae used in this study were defined as larvae due to the presence of gills, tail fins, and their completely aquatic nature. Adults of the species lack the gills and tail fins of the post hatching larval stage, and are primarily terrestrial and fossorial as well (Dent, 1968).

No attempt was made to determine the age of the larval *Ambystoma tigrinum* because age-determining techniques for this species are unknown. Individuals used in this study were estimated to be in excess of two years of age because of their large size (20 to 30 cm) and their grey coloration. An abundance of small (2 to 6 cm) yellow-green semitransparent larvae were netted in Tyrrell's Lake during June and July, 1970. These were assumed to be the larvae of that year's spawn. Toward the end of September and beginning of October the smallest *Ambystoma tigrinum* larvae caught were between 10 and 15 cm long and were a green-grey with some loss of the transparent nature of the tail fin and skin. All individuals older than the first summer-winter period have a grey appearance and appear alike except for size and scars. The presumably older animals are large (often in excess of 30 cm) and heavily scarred. Gill filaments were often missing and tail fins were often torn and uneven.

Since the *Ambystoma tigrinum* used were estimated to be in excess of one to two years of age, they can be considered as neotenic. Neoteny in amphibians only implies an extended larval life and does not reflect

on the ability of the larvae to reproduce sexually (Smith, 1969).

A group of large larvae trapped in May of 1970 spawned in the transporting tanks as well as later in the holding tanks. A group of their eggs was removed from the holding tank and placed in a large finger bowl at room temperature (20°C). They were observed for a period of six days before they were attacked by fungus. Cleavage occurred and the eggs were therefore assumed to be fertile. This would seem to indicate the larvae were sexually mature and capable of reproducing. On this basis, these larvae are classified as paedogenetic (Dent, 1968).

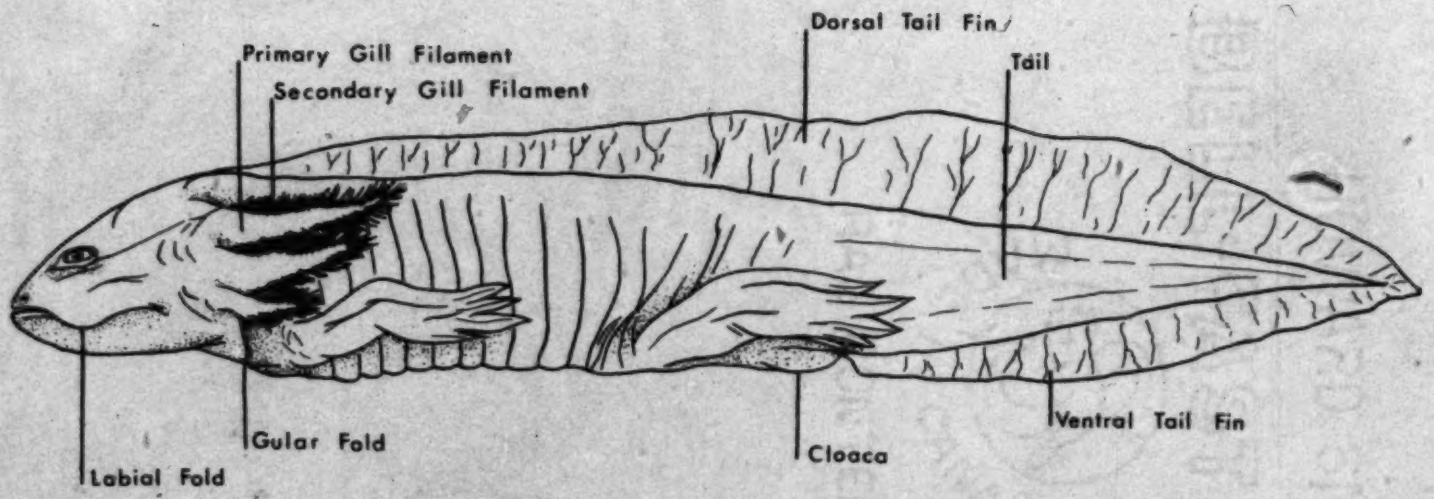
Staging of Metamorphosis in *Ambystoma tigrinum*

Preliminary observations on the general sequence of metamorphic events and the subsequent study of both fixed and living, partially metamorphosed larvae led to the definition of ~~six~~ stages, each based on external characteristics. The presence or absence of various characteristics used for determining the stages of hormonally induced metamorphosis included the pelvic and pectoral appendages, the dorsal and ventral tail fins, the labial folds, the gular folds, gills and gill slits, and the eyelids.

Stage I

The premetamorphic larva of *Ambystoma tigrinum* is well equipped for the aquatic habitat. The pectoral and pelvic limbs are unmuscular, flat and fleshy in appearance, with broad, flat blade-like digits (Fig. 5, Plates 2 and 3). The pelvic limbs are much broader than the pectoral

Fig. 5. Lateral view of *Ambystoma tigrinum* Stage I-(larva).


Primary Gill Filament
Secondary Gill Filament
Dorsal Tail Fin
Tail
Labial Fold
Gular Fold
Cloaca
Ventral Tail Fin
1 cm.

ones due to a large dorsal fleshy lobe. Both the dorsal and ventral tail fins are well developed. The dorsal tail fin arises from a broad base beginning just posterior to the head region and extending posteriorly to the tip of the muscular tail. The crest of the fin is thin and uneven, with the greatest depth just dorsal to the cloaca and the least depth just posterior of head region and at the tip of the muscular tail. The ventral tail fin arises from a thick base just posterior to the cloaca and extends posteriorly to the tip of the muscular tail to meet the dorsal tail fin. The ventral tail fin rises sharply to a crest just posterior of the cloaca. The crest is thin and uneven with its greatest depth midway between the cloaca and the tip of the muscular tail, where its least depth occurs.

The labial folds at the corner of the mouth are prominent and well developed (Fig. 6). The gular fold is thin and semitransparent with a prominent medial slit. It is not attached at its posterior margin at this stage.

The gills of the premetamorphic larva are a prominent feature of this species. The three long (unbranched) primary gill filaments arise from thick bases at the latero-posterior head region and taper gradually to points posterior (Fig. 7). The long dense blade-like secondary gill filaments branch directly off the primary gill filaments along their length. The secondary gill filaments are heavily pigmented.

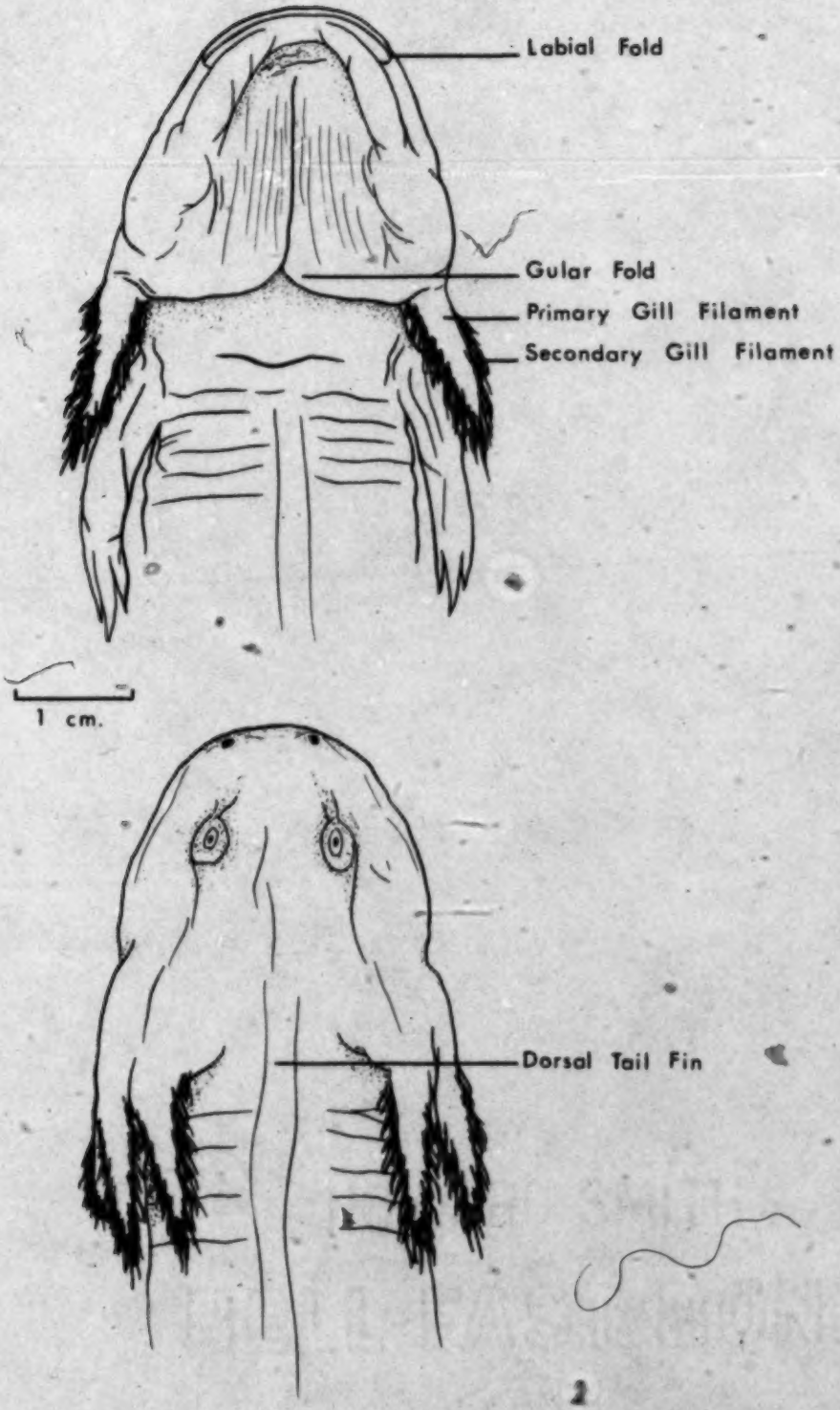
The color of the larva varies with age and at this stage the color was generally grey or green-grey.

Stage II

With the onset of induced metamorphosis, the second stage of

Fig. 6. Ventral view of *Ambystoma tigrinum* Stage I (larva).

Fig. 7. Dorsal view of *Ambystoma tigrinum* head Stage I (larva).


Labial Fold
Gular Fold
Primary Gill Filament
Secondary Gill Filament
1 cm.
Dorsal Tail Fin

of metamorphosis can be recognized.

The pelvic and pectoral appendages are less fleshy and more muscular in appearance (Fig. 8). The dorsal fleshy lobe of the pelvic appendage is absent. The digits of both pelvic and pectoral appendages are reduced in width and assume the adult form.

There is a general reduction in the size of both the dorsal and ventral tail fins due to absorption. The base of the anterior portion of the dorsal tail fin is reduced in width laterally and the depth (dorsal-ventral) of the crest in the head region is greatly reduced. There is a general thickening and decreased depth in the crest of the dorsal tail fin along its length. The base of the anterior portion of the ventral tail fin is reduced in width laterally and the high crest at the posterior margin of the cloaca is absent. There is a general thickening and decreased depth of the crest of the ventral tail fin along its length.

The labial folds are present but greatly reduced in size (Fig. 9). The posterior margin of the gular fold is thickened and the medial slit is absent. It is not attached to the trunk at its posterior margin.

There is a reduction in the width and thickness but not the length of the primary gill filaments (Fig. 10). The secondary gill filaments are reduced in length and width but not in number.

Stage III

The pelvic and pectoral appendages of the Stage III larva are slightly more muscular in appearance and differ little, if any, from the adult form.

Both dorsal and ventral tail fins are greatly reduced (Fig. 11).

Fig. 8. Lateral view of *Ambystoma tigrinum* Stage II.

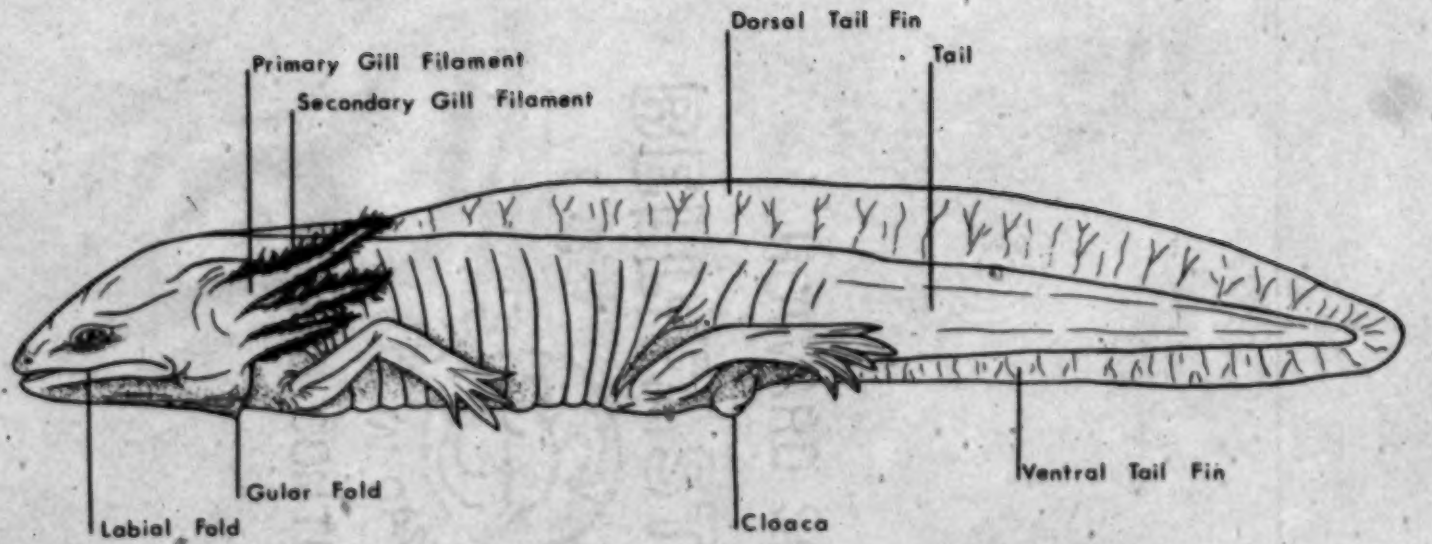
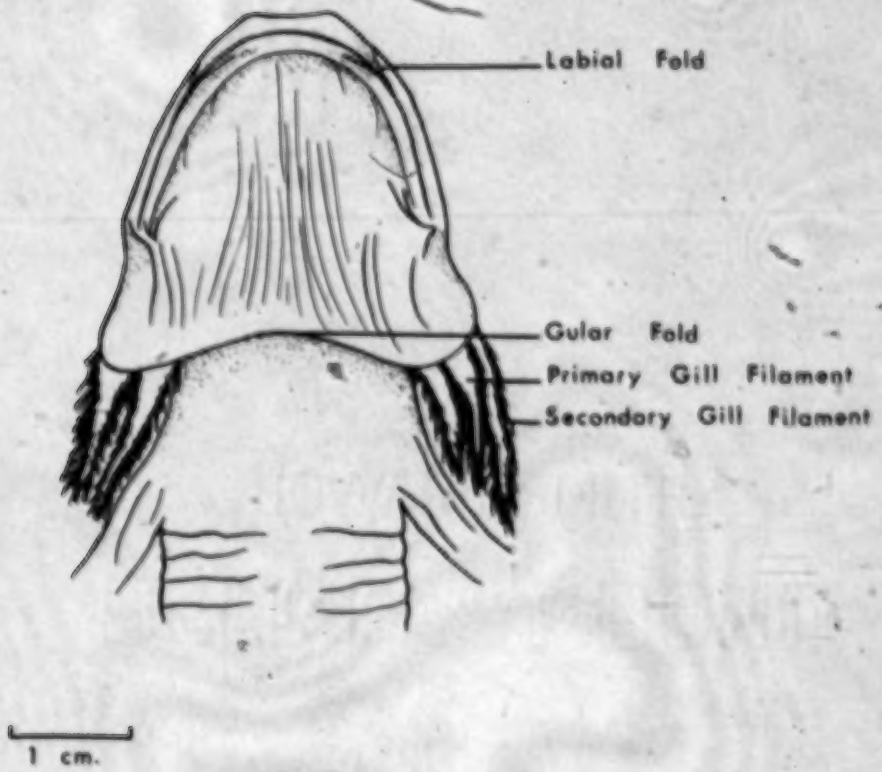
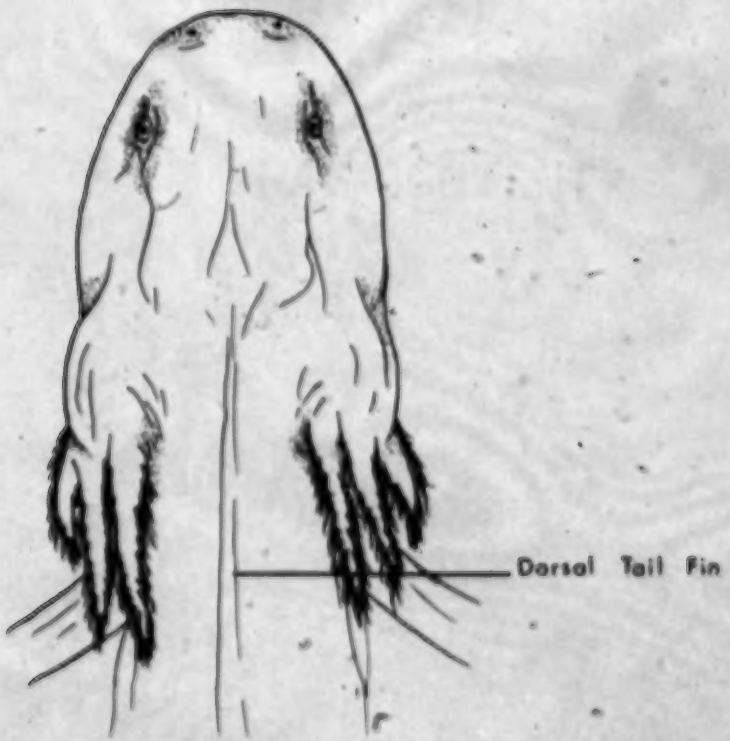

Dorsal Tail Fin
Tail
Primary Gill Filament
Secondary Gill Filament
Ventral Tail Fin
Gular Fold
Cloaca
Labial Fold
1 cm.

Fig. 9. Ventral view of *Ambystoma tigrinum* head Stage II.

Fig. 10. Dorsal view of *Ambystoma tigrinum* head Stage II.


Labial Fold
Gular Fold
Primary Gill Filament
Secondary Gill Filament
1 cm.


Dorsal Tail Fin

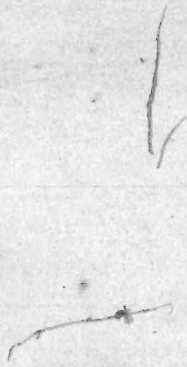
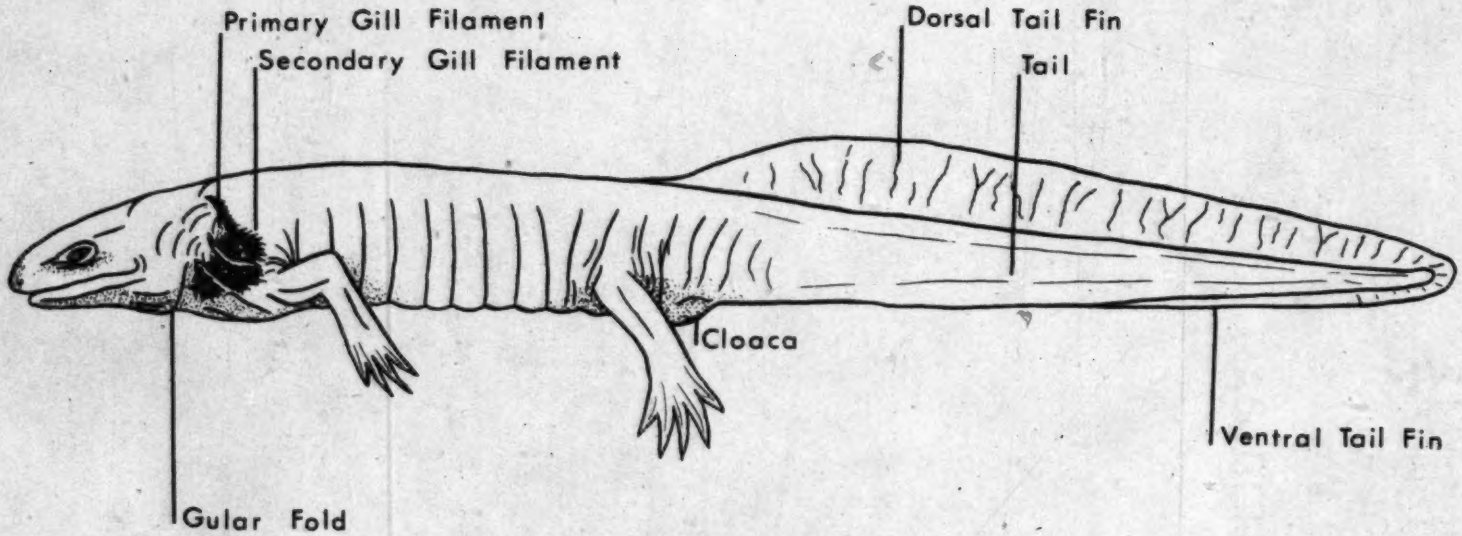


Fig. 11. Lateral view of *Ambystoma tigrinum* Stage III.


Primary Gill Filament
Secondary Gill Filament
Dorsal Tail Fin
Tail
Cloaca
Ventral Tail Fin
Gular Fold
1 cm.

The dorsal tail fin has been absorbed posteriorly to a point just dorsal of the cloaca. A heavily pigmented (black) scar is present in the region of the absorbed anterior portion. The ventral tail fin has been absorbed leaving only a small portion in the distal tail region.

The labial folds are absent (Fig. 12). The gular fold is very thick and ridged but is not yet fused to the trunk region.

The gills are greatly reduced in size, with only short, curled and heavily pigmented primary gill filaments. Secondary gill filaments (Fig. 13) are present but greatly reduced in number and size.

Eyelid development is first evident at this stage but they are not as yet fully developed.

Stage IV

In Stage IV the dorsal tail fin is absorbed to a point posterior to the cloaca, leaving only a small portion in the distal tail region (Fig. 14). The ventral tail fin is only represented by a trace amount in the distal tail region. In both the dorsal and ventral tail regions where tail fins have been absorbed there remains a heavily pigmented tail fin scar.

The gular fold is fused at its center to the trunk, slightly anterior to the margin (Fig. 15). The margin is thick and very ridged.

The primary filaments of the gills are reduced to heavily pigmented stumps and the secondary gill filaments are absent (Fig. 16). The first two of the three gill slits are fused shut.

Eyelids are fully developed at this stage. The adult color pattern can be recognized in some larvae but this varies greatly with the individual at this stage.

Fig. 12. Ventral view of *Ambystoma tigrinum* head Stage III.

Fig. 13. Dorsal view of *Ambystoma tigrinum* head Stage III.

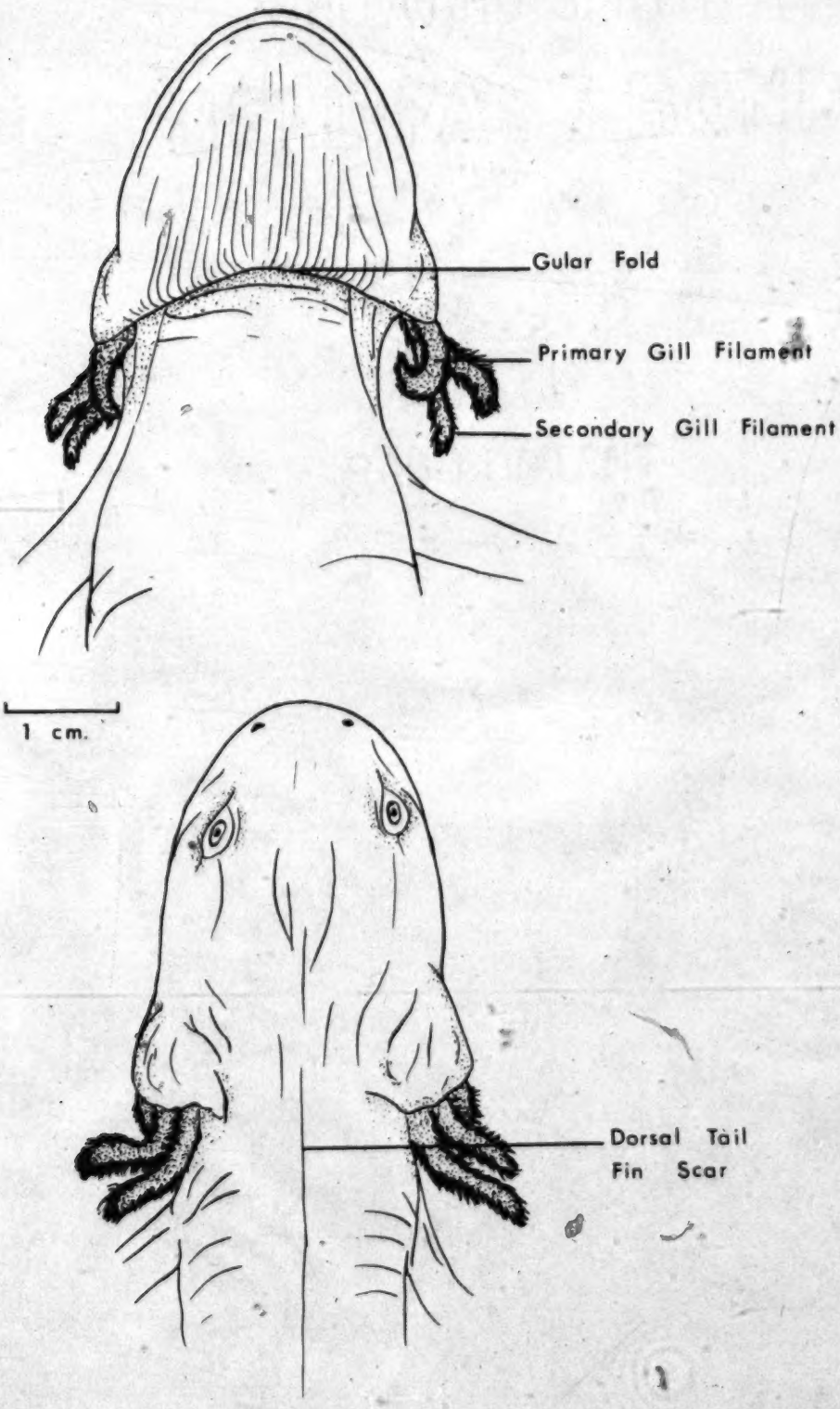

Gular Fold
Primary Gill Filament
Secondary Gill Filament
1 cm.
Dorsal Tail
Fin Scar

Fig. 14. Lateral view of *Ambystoma tigrinum* Stage IV.

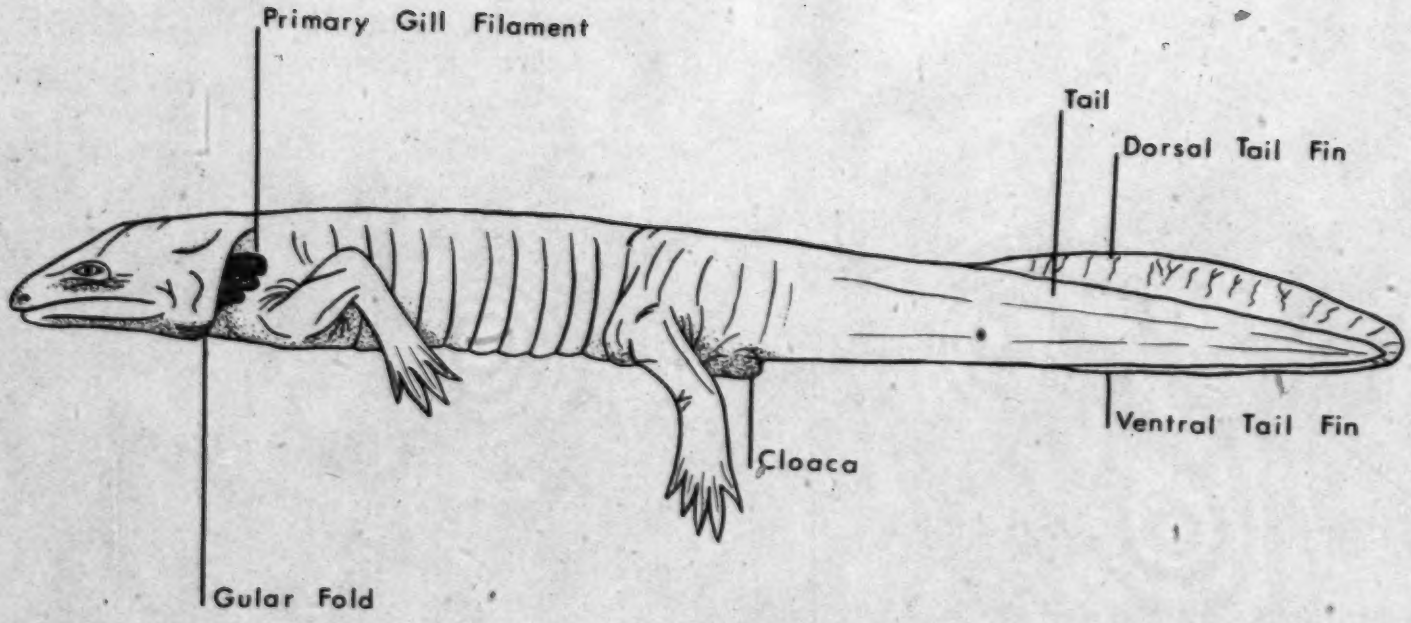
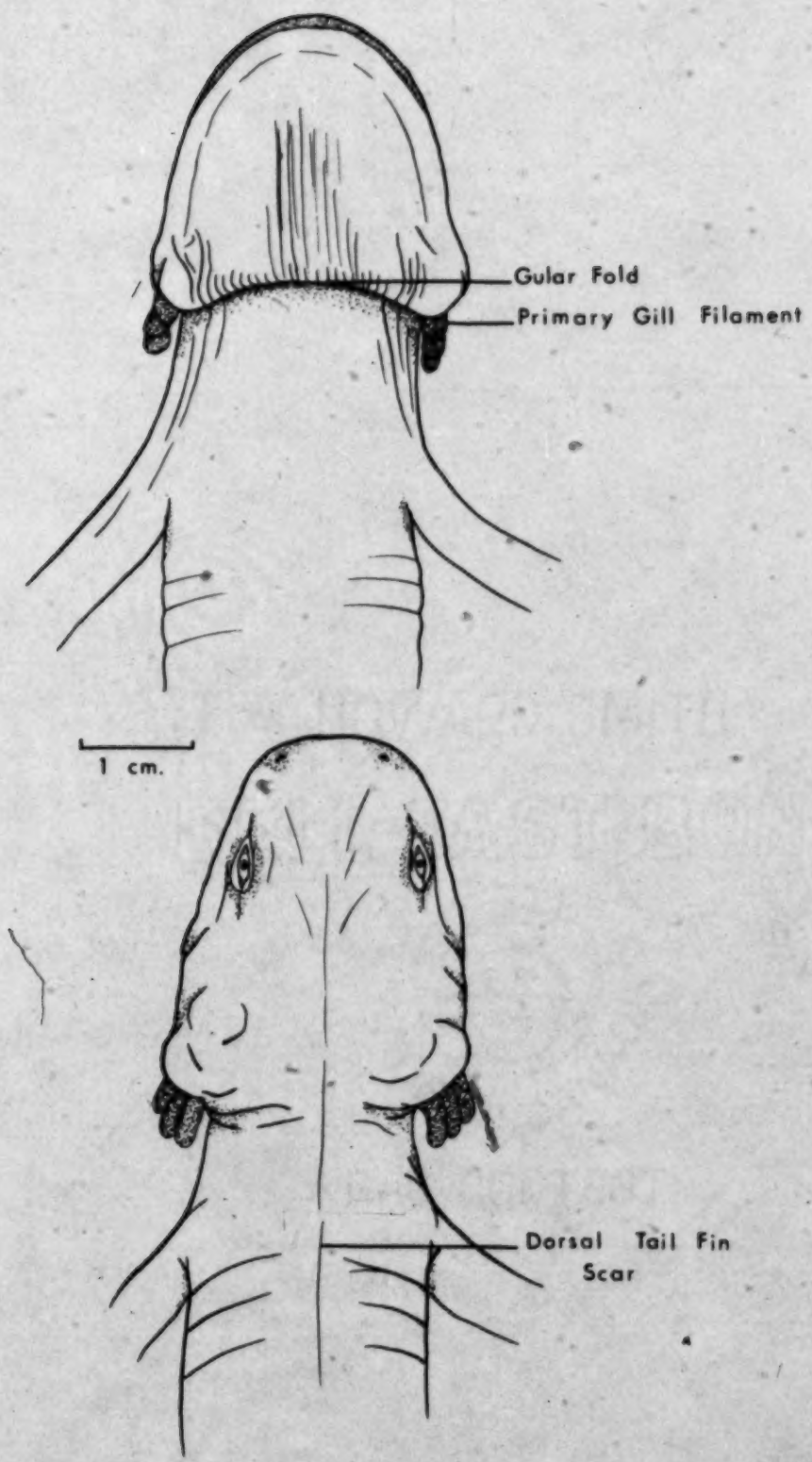

Primary Gill Filament
Tail
Dorsal Tail Fin
Ventral Tail Fin
Cloaca
Gular Fold
1 cm.

Fig. 15. Ventral view of *Ambystoma tigrinum* head Stage IV.

Fig. 16. Dorsal view of *Ambystoma tigrinum* head Stage IV.


Gular Fold
Primary Gill Filament
1 cm.
Dorsal Tail Fin
Scar

Stage V

The dorsal and ventral tail fins are both absent at this stage leaving only heavily pigmented tail fin scars (Fig. 17, Plates 4 and 5).

The primary gill filaments are absent leaving only heavily pigmented gill scars (Figs. 18 and 19). There is evidence of unabsorbed hypobranchial apparatus in the region of the pigmented gill scars. The last gill slit is closed.

The gular fold is fused along its entire margin at this stage but remains very thick and ridged (see Fig. 18).

The larval coloration of grey or green-grey is replaced by the adult pigmentation of black and yellow.

Stage VI

This stage represents the adult form of *Ambystoma tigrinum* after the completion of metamorphosis (Figs. 20, 21 and 22). Tail fin scars are absent as are the gill scars (see Fig. 20). The gular fold is represented by only a small crease on the ventral neck region (see Fig. 21). The tail is blunt and muscular.

Thyroxine Experiments

a) Experiment 1

All concentrations of T_4 used caused the initiation of metamorphosis at 15 and 20°C (Table 1). All larvae in the control groups at 15 and 20°C remained in Stage I and showed no signs of metamorphosis.

The mean weight loss per animal per day was calculated (Table 2) to determine the relative effect on the rate of metamorphosis by the different concentrations of T_4 used. No significant difference in the mean weight

Fig. 17. Lateral view of *Ambystoma tigrinum* Stage V.

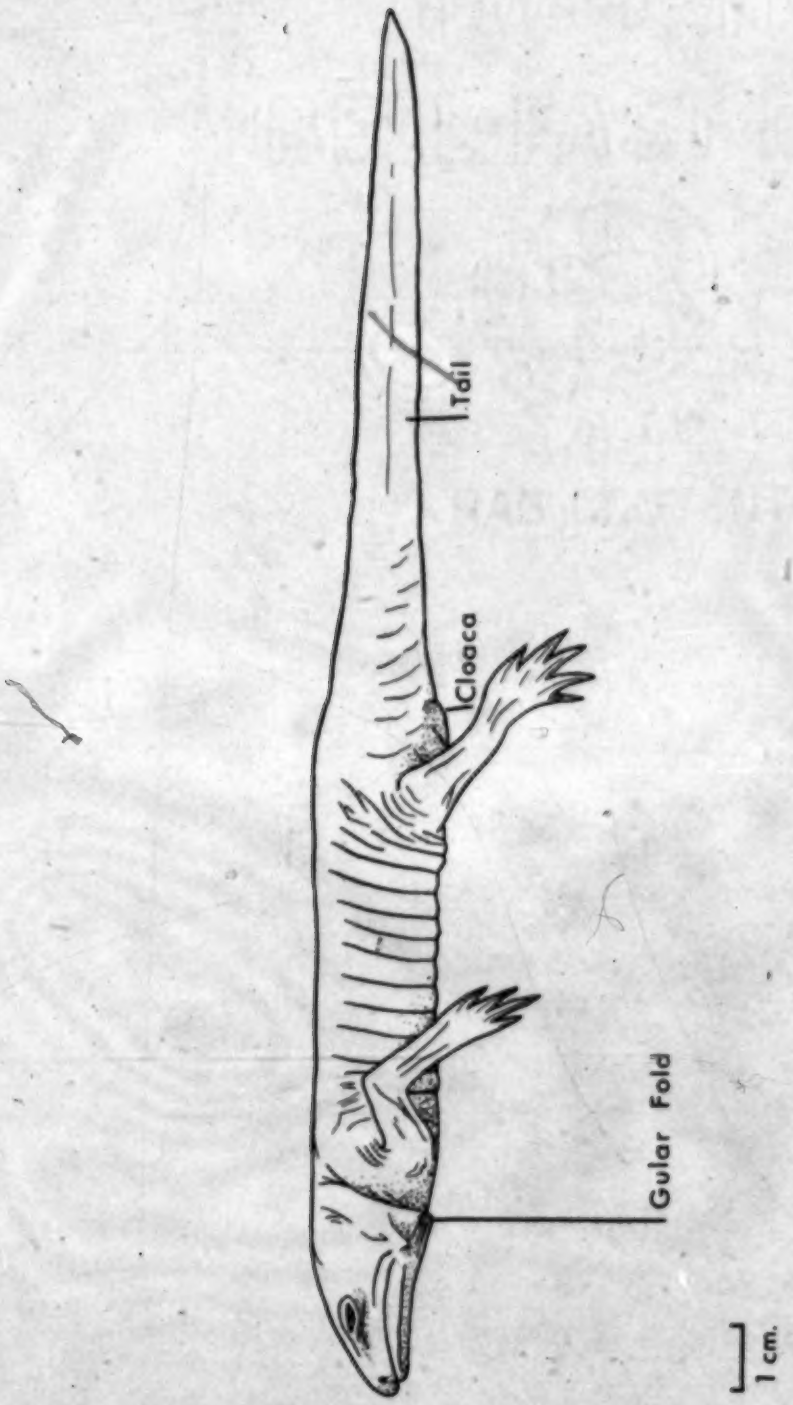

Tail
Cloaca
Gular Fold
1 cm.

Fig. 18. Ventral view of *Ambystoma tigrinum* head Stage V.

Fig. 19. Dorsal view of *Ambystoma tigrinum* head Stage V.

Gill Scars
Gular Fold
1 cm.
Dorsal Tail Fin
Scar

Fig. 20. Lateral view of *Ambystoma tigrinum* Stage VI (adult).

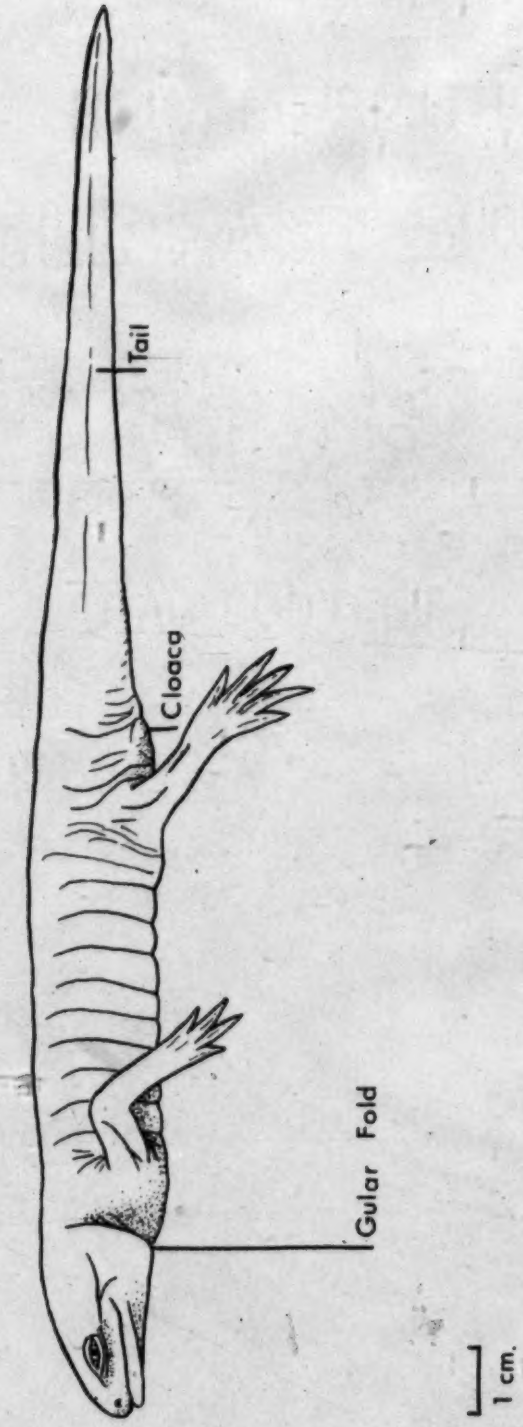
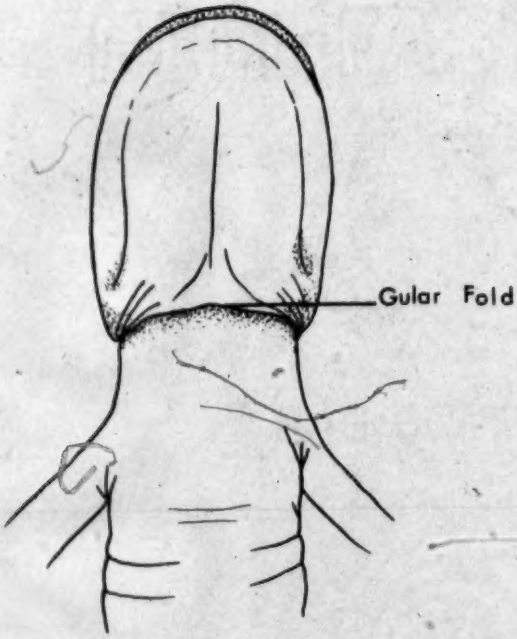
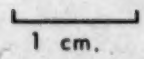

Tail
Cloaca
Gular Fold
1 cm.

Fig. 21. Ventral view of *Ambystoma tigrinum* head Stage VI (adult).

Fig. 22. Dorsal view of *Ambystoma tigrinum* head Stage VI (adult).


Gular Fold


1 cm.

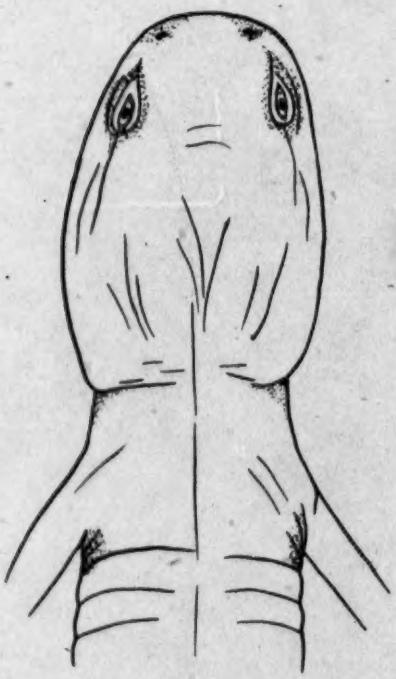


Table 1

THE EFFECT OF L-THYROXINE AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM**

Temperature in Degrees Centigrade	Micrograms of L-thyroxine Injected per Gram Net Weight	Number of Injections	Number of Animals Initiating Metamorphosis	Number of Animals Unmetamorphosed	Mortality
15	4.0	1	5	0	0
	1.0	1	5	0	0
	0.25	1	5	0	0
	0.0625	1	5	0	0
	0.0	1	0	5	0
20	4.0	1	5	0	0
	1.0	1	5	0	0
	0.25	1	5	0	0
	0.0625	1	5	0	0
	0.0	1	0	5	0

*The period of observation post-injection was 14 days.

Table 2

MEAN WEIGHT LOSS (IN GRAMS) PER DAY IN *AMBYSTOMA TIGRINUM* AFTER INJECTION WITH VARIOUS DOSES OF L-THYROXINE (n = 5)

Temperature in Degrees Centigrade	Micrograms L-thyroxine Injected per Gram Wet Weight				
	4.0	1.0	0.25	0.0625	0.0
20	1.788	1.205	2.153	2.005	0.277
	3.110	0.976	1.516	2.329	1.016
	1.561	1.229	2.166	1.222	0.838
	0.900	1.047	2.392	1.527	0.055
	1.500	1.688	1.388	1.476	0.438
15	1.964	1.888	0.850	1.488	0.216
	0.650	1.516	0.572	1.277	0.572
	1.900	1.317	1.405	1.444	0.305
	1.261	1.394	1.333	1.405	0.250
	1.720	0.982	1.333	0.411	0.722

loss was found within the replicates ($f = 0.5453$, d.f. = 36.4, $p < 0.01$) but a significant difference was found between the treatments (T_4 injections) ($f = 9.929$, d.f. = 36.4, $p < 0.01$). There was no significant temperature-treatment interaction ($f = 1.693$, d.f. = 36.4, $p < 0.01$). Therefore the amount of T_4 injected was an important factor for all further induced-metamorphosis studies. Since the mean weight loss is not necessarily an important parameter but rather only an indication of the relative activity of T_4, the difference in the means of the treatments was not used. Instead, an arbitrary injection strength of 1 µgm T_4 per gram wet weight of animal was chosen from the treatment concentrations to use in future studies.

b) Experiment 2

Since experiment one demonstrated the capacity of the *Ambystoma tigrinum* larvae to respond to T_4 it was necessary to determine if the larvae were capable of completing metamorphosis and the effect of temperature on this process. At temperatures of 15, 20 and 25°C the *Ambystoma tigrinum* larvae receiving 1 µg T_4 per gram wet weight exhibited the ability to complete metamorphosis (Table 3) with the exception of three individuals at 15°C, one individual at 20°C and two individuals at 25°C. Larvae held at a temperature of 10°C remained at Stage I and did not metamorphose.

Temperature Experiments

a) Experiment 1

Although reports in the literature suggest that temperature may be a factor in causing the onset of metamorphosis (Smith, 1969), the results

Table 3

THE EFFECT OF TEMPERATURE ON THE L-THYROXINE INDUCED METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*[*]

Number of Animals Used	Type of Injection	Temperature in Degrees Centigrade	Number of Animals Metamorphosed to Stage VI	Number of Animals Unmetamorphosed	Mortality	Number of Days Required to Reach Stage VI
24	sham	10	0	22	2	—
24	L-thyroxine	10	0	20	4	—
24	sham	15	0	19	5	—
24	L-thyroxine	15	13	3	8	46
24	sham	20	0	17	7	—
24	L-thyroxine	20	12	1	11	32
24	sham	25	0	18	6	—
24	L-thyroxine	25	13	2	9	30

*The period of observation was 46 days.

of the thyroxine-temperature experiments, as summarized in Tables 1—6 (by observation of sham injected larvae) and specifically Table 6, do not demonstrate this. There was no sign of metamorphosis in any of the 40 *Ambystoma tigrinum* larvae at 10, 15, 20 and 25°C. All the larvae remained in Stage I during the 21 days of observation (Table 6). All larvae appeared healthy at the termination of the experiment and no fungus infections were evident.

b) Experiment 2

This experiment was similar to the preceding experiments in that sham injected controls were observed for signs of metamorphosis but for a period of 46 days. All the controls remained at Stage I during the experiment although there was some loss due to fungus infection (Tables 3, 5) in the controls as well as in the T_4 injected larvae. The daily mortality of the controls and experimentals is summarized in Appendix IV.

The effect of temperature on the rate of thyroxine induced metamorphosis is shown in Table 4 and Figure 23. As would be expected, the rate of metamorphosis increases with increased temperature. Individuals kept at 10°C remained in Stage I and showed no signs of metamorphosis.

Iodide Experiments

In combination with temperatures of 15, 20 and 25°C (those temperatures at which the larva have been shown to have been capable of undergoing T_4 induced metamorphosis), the various concentrations of potassium iodide (Table 7) had no effect on the induction of metamorphosis in the larvae tested. All the larvae remained at Stage I during the three weeks of observation, showing no signs of metamorphosis. There was no death

Table 4

THE EFFECT OF TEMPERATURE ON THE RATE OF METAMORPHOSIS OF L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM*

Temperature in Degrees Centigrade	Number of Animals in Group	Stage in Metamorphosis	First Day that Stage Characteristics Appeared in Larvae of the Group	Last Day that Stage Characteristics Appeared in Larvae of the Group	Mean Day that Stage Characteristics Appeared in Larvae of the Group
25	24	I	0	0	0
		II	3	5	4
		III	5	7	6
		IV	7	9	8
		V	12	16	14
		VI	28	32	30
20	24	I	0	0	0
		II	5	7	6
		III	8	10	9
		IV	11	13	12
		V	16	20	18
		VI	30	34	32
15	24	I	0	0	0
		II	10	12	11
		III	13	15	14
		IV	16	20	18
		V	25	29	27
		VI	42	50	46
10	24	I—VI	0	0	0

Table 5

THE MORTALITY IN SHAM AND L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM* LARVAE AT 10, 15, 20 and 25° CENTIGRADE OVER A PERIOD OF 46 DAYS

Type of Injection on Day 0	Temperature in Degrees Centigrade	Time in Days from Injection							
		0—5	6—10	11—15	16—20	21—25	26—30	31—35	36—46
Sham	10	1	1	0	0	0	0	0	0
L-thyroxine	10	1	0	0	0	0	3	0	0
Sham	15	0	3	1	1	0	0	0	0
L-thyroxine	15	0	4	0	0	1	1	2	0
Sham	20	3	0	1	2	0	1	0	0
L-thyroxine	20	2	5	3	0	0	1	0	0
Sham	25	1	0	1	2	2	0	0	0
L-thyroxine	25	0	6	2	0	1	0	0	0

Table 6

THE EFFECT OF TEMPERATURE AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM*

Temperature in Degrees Centigrade	Number of Animals Metamorphosed	Number of Animals Unmetamorphosed	Mortality
10	0	10	0
15	0	10	0
20	0	10	0
25	0	10	0

Fig. 23. The influence of temperature on the T_4 induced metamorphosis of *Ambystoma tigrinum* larvae. Symbols indicate means and bars indicate first and last days that stage characteristics appeared.

● - 10°C ● - 15°C ■ - 20°C ▲ - 25°C

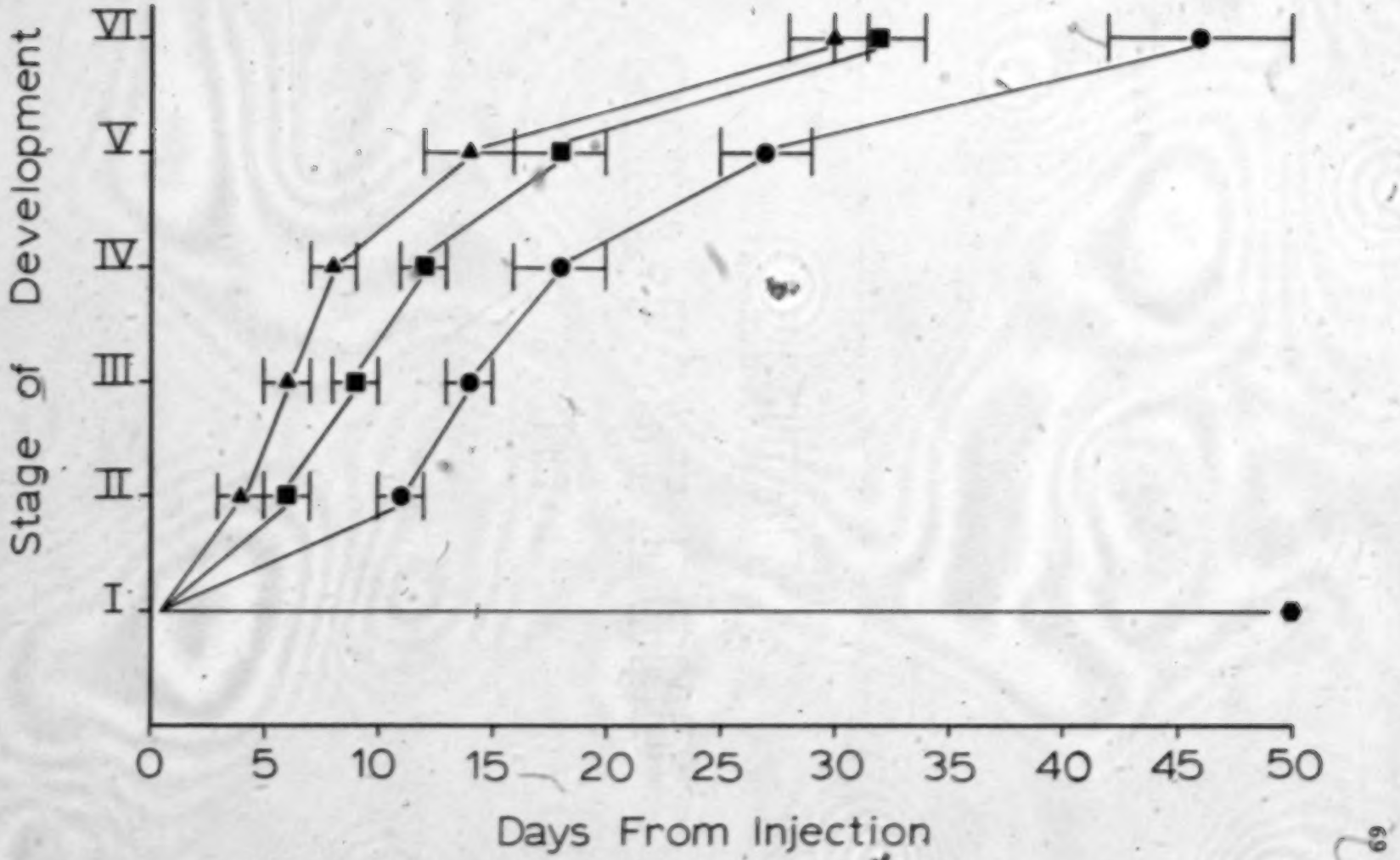

Stage of Development
VI
V
IV
III
II
I
0
5
10
15
20
25
30
35
40
45
50
Days From Injection

Table 7

THE EFFECT OF POTASSIUM IODIDE AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM**

Temperature in Degrees Centigrade	Micrograms of Potassium Iodide per Liter of Water	Number of Animals Initiating Metamorphosis	Number of Animals Unmetamorphosed	Mortality
15	40	0	10	0
	20	0	10	0
	5	0	10	0
	0	0	10	0
20	40	0	10	0
	20	0	10	0
	5	0	10	0
	0	0	10	0
25	40	0	10	0
	20	0	10	0
	5	0	10	0
	0	0	10	0

*The period of observation was 21 days.

due to fungal infection and all larvae appeared in good health after the 3-week period.

Thyroid Stimulating Hormone Experiments

a) Experiment 1

Single injections of bovine TSH at two temperatures (20 and 25°C) gave varied results (Table 8). In the groups of larva given 1.0 and 0.5 I.U. of TSH, metamorphosis was induced in only seven individuals out of a total of 18 (Table 8). At a concentration of 0.25 I.U. of TSH, metamorphosis was not induced in any of the *Ambystoma tigrinum* larvae, and all remained at Stage I during the two weeks of observation. There was some mortality in the group at 25°C (two) (Table 8) due to fungal infection.

b) Experiment 2

In the second TSH experiment, single injections of 1.0 I.U. of TSH alone and in combination with potassium iodide were used with similar results to that in Experiment 1 (Table 9). Induction of metamorphosis was observed in 12 *Ambystoma tigrinum* larvae (six in the potassium iodide treated groups [at 20 and 25°C] and six in the groups without potassium iodide [at 20 and 25°C]) although there were individuals that showed no response to the injections of TSH and remained at Stage I (six) (Table 9) over the two weeks of observation. There was some mortality due to fungal infection (Table 9) and it was not certain if the dead animals were responding to the TSH injections.

c) Experiment 3

Experiment 3 was designed similarly to Experiment 2, but here the

Table 8

THE EFFECT OF THYROID STIMULATING HORMONE AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM**

Temperature in Degrees Centigrade	International Units of TSH per Injection	Number of Injections	Number of Animals Initiating Metamorphosis	Number of Animals Unmetamorphosed	Mortality
20	1.0	1	2	3	0
	0.5	1	1	4	0
	0.25	1	0	5	0
	0.0	1	0	5	0
25	1.0	1	3	1	1
	0.5	1	1	3	1
	0.25	1	0	5	0
	0.0	1	0	5	0

*The period of observation was 14 days.

Table 9

THE EFFECT OF A SINGLE INJECTION OF THYROID STIMULATING HORMONE IN COMBINATION WITH IODIDE AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM**

Temperature in Degrees Centigrade	International Units of TSH per Injection	Micrograms of Potassium Iodide per Liter of Water	Number of Animals Metamorphosed	Number of Animals Unmetamorphosed	Mortality
	1.0	40	3	1	1**
20	1.0	0	2	3	0
	0.0	0	0	5	0
	1.0	40	3	1	1**
25	1.0	0	4	1	0
	0.0	0	0	5	0

*The period of observation was 14 days.

**Mortality occurred before it could be determined in which group (metamorphosis initiated or unmetamorphosed) the animals belonged.

larvae were given multiple injections of TSH alone or in combination with potassium iodide (Table 10). The induction of metamorphosis occurred in all individuals (in both temperatures of 20 and 25°C) given multiple injections of TSH whether alone or in combination with potassium iodide. There was no mortality due to fungal infections, although its presence was evident on many of the animals toward the end of the 2-week observation.

Oxygen Consumption Experiments

a) Experiment 1

From Table 11 it can be seen that the mean oxygen consumption of 40 sham injected *Ambystoma tigrinum* larvae (mean oxygen consumption = 0.598 mg O_2 per gram dry weight per hour ± S.E.M. 0.0390) is significantly higher than the mean of the same 40 larvae treated with 1 µgm per gram wet weight T_4 (mean oxygen consumption = 0.484 mg O_2 per gram dry weight per hour ± S.E.M. 0.0288) (t = 2.336, d.f. = 78, $p < 0.05$). The thyroxine given to the larvae has a depressing effect on the rate of oxygen uptake at one hour post injection. The means of the dry weights at one hour post injection were not significantly different ($p > 0.05$), as would be expected (Appendix V).

b) Experiment 2

In 20 of the larvae used in Experiment 1, the oxygen consumption was also measured at 24 hours post injection (24 hours from the T_4 injection of Experiment 1) and compared to the one hour post injection oxygen consumption results from the above experiment. This would determine if the depressing effect of thyroxine on the oxygen consumption is

Table 10

THE EFFECT OF MULTIPLE INJECTIONS (ONE DAILY FOR SIX DAYS) OF THYROID STIMULATING HORMONE IN COMBINATION WITH IODIDE, AS A METAMORPHOSING AGENT IN *AMBYSTOMA TIGRINUM**

Temperature in Degrees Centigrade	International Units of TSH per Injection	Micrograms of Potassium Iodide per Liter of Water	Number of Animals Initiating Metamorphosis	Number of Animals Unmetamorphosed	Mortality
	1.0	40	5	0	0
20	1.0	0	5	0	0
	0.0	0	0	5	0
	1.0	40	5	0	0
25	1.0	0	5	0	0
	0.0	0	0	5	0

*The period of observation was 14 days.

Table 11

INDIVIDUAL OXYGEN CONSUMPTIONS IN SHAM AND L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM* ONE HOUR POST INJECTION (Ta = 15°C)

Milligrams of Oxygen Consumed per Gram Dry Weight per Hour	
Sham Injected	*L-thyroxine Injected*
0.448	0.542
0.690	0.854
0.773	0.468
0.677	0.871
1.080	0.568
1.061	0.263
0.657	0.311
0.638	0.408
0.180	0.278
0.092	0.325
0.435	0.542
0.496	0.429
0.307	0.341
0.620	0.287
0.480	0.431
0.912	0.540
0.800	0.412
0.887	0.513
0.486	0.386
0.617	0.383
0.716	0.350
0.760	0.849
0.752	0.522
0.302	0.635
0.554	0.314
0.745	0.513
0.607	0.541
0.675	0.912
0.127	0.205
0.371	0.397
0.351	0.608
0.086	0.905
0.425	0.330
0.671	0.277
0.623	0.584
0.400	0.324
0.849	0.482
0.902	0.574
0.864	0.448
0.784	0.444
$\bar{X}$ ± S.E.M.	$\bar{X}$ ± S.E.M.
0.598 ± 0.0390	0.484 ± 0.0288

Note: For animal dry weights see Appendix V.

just transitory. The results (Table 12) show the continuing trend as demonstrated in Experiment 1. The mean oxygen consumption of the larvae injected with T_4 at one hour post injection (mean oxygen consumption = 0.454 mg O_2 per gram dry weight per hour ± S.E.M. 0.0425) was significantly higher than the mean of the 24 hour post injected larvae (mean oxygen consumption = 0.339 mg O_2 per gram dry weight per hour ± S.E.M. 0.0264) ($t = 2.307$, d.f. = 38, $p < 0.05$).

c) Experiment 3

There appears to be a depression of the oxygen consumption exhibited by injections of thyroxine as demonstrated in Table 13 and Figure 24. The values in the table are the mean oxygen consumption of four individuals except in the case of Day 4 and Day 7 where the mean represents that of only two larvae. The oxygen consumption values of Day 7 sham injected larvae are omitted from Figure 24 because there was some trouble with the respirometer due to water leaks that were not observed until after the start of the experimental run.

Wet and Dry Weight Determinations

The wet and dry weights (Table 14) of the larval *Ambystoma tigrinum* are plotted in Figure 25 using a regression equation. A program and regression equation were constructed from this data to calculate a predicted dry weight for any observed wet weight. This program was incorporated into the program that calculated the oxygen consumption (changing the observed wet weight of an *Ambystoma tigrinum* larva to a predicted dry weight) so that values of oxygen consumption would come out as mg O_2 consumed per gram dry weight per hour.

Table 12

INDIVIDUAL OXYGEN CONSUMPTION IN L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM*

Time of Measurement in Hours from Injection of L-thyroxine (1 µg per gram wet weight)			
Milligrams of Oxygen Consumed per Gram Dry Weight per Hour		Animal Dry Weight in Grams*	
1	24	1	24
0.522	0.194	17.029	17.325
0.468	0.516	17.177	15.401
0.635	0.143	14.142	26.282
0.871	0.462	18.732	15.475
0.3	0.471	15.253	14.883
0.568	0.194	19.102	13.328
0.513	0.286	19.546	15.993
0.263	0.266	20.138	17.695
0.205	0.176	13.328	12.514
0.278	0.194	17.325	14.216
0.397	0.289	12.588	12.292
0.325	0.469	16.659	13.550
0.609	0.508	15.919	13.328
0.542	0.339	15.031	10.959
0.905	0.332	14.216	15.623
0.429	0.364	12.588	10.663
0.330	0.319	13.328	19.398
0.342	0.341	16.437	15.697
0.277	0.488	13.920	18.880
0.287	0.423	11.329	18.806
$\bar{X}$ ± S.E.M.	$\bar{X}$ ± S.E.M.	$\bar{X}$ ± S.E.M.	$\bar{X}$ ± S.E.M.
0.454 ± 0.0425	0.339 ± 0.0264	15.679 ± 0.555	16.311 ± 1.162

*Determined from regression equation.

Table 13

MEAN OXYGEN CONSUMPTION IN MILLIGRAMS OXYGEN CONSUMED PER GRAM DRY WEIGHT PER HOUR IN FOUR SHAM INJECTED AND FOUR L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM*

Type of Injection		Day from Injection of L-thyroxine or Sham									
		0	1	2	3	4	5	6	7*	8	9
sham	$\bar{X}$	0.500	0.705	0.549	0.568	0.553	0.583	0.763	0.275	0.509	0.573
	S.E.M.	± .08	± .05	± .09	± .03	± .08	± .07	± .09		± .02	± .07
L-thyroxine	$\bar{X}$	0.596	0.441	0.369	0.431	0.646	0.516	0.631	0.434	0.396	0.659
	S.E.M.	± .05	± .07	± .06	± .06	± .09	± .10	± .07	± .05	± .01	± .02

*See *c) Experiment 3* preceding.

Fig. 24. The mean oxygen consumption of T_4 and sham injected *Ambystoma tigrinum* larvae over a 10-day period. (▲ T_4 injected, ■ sham injected ± 1 S.E.M.).

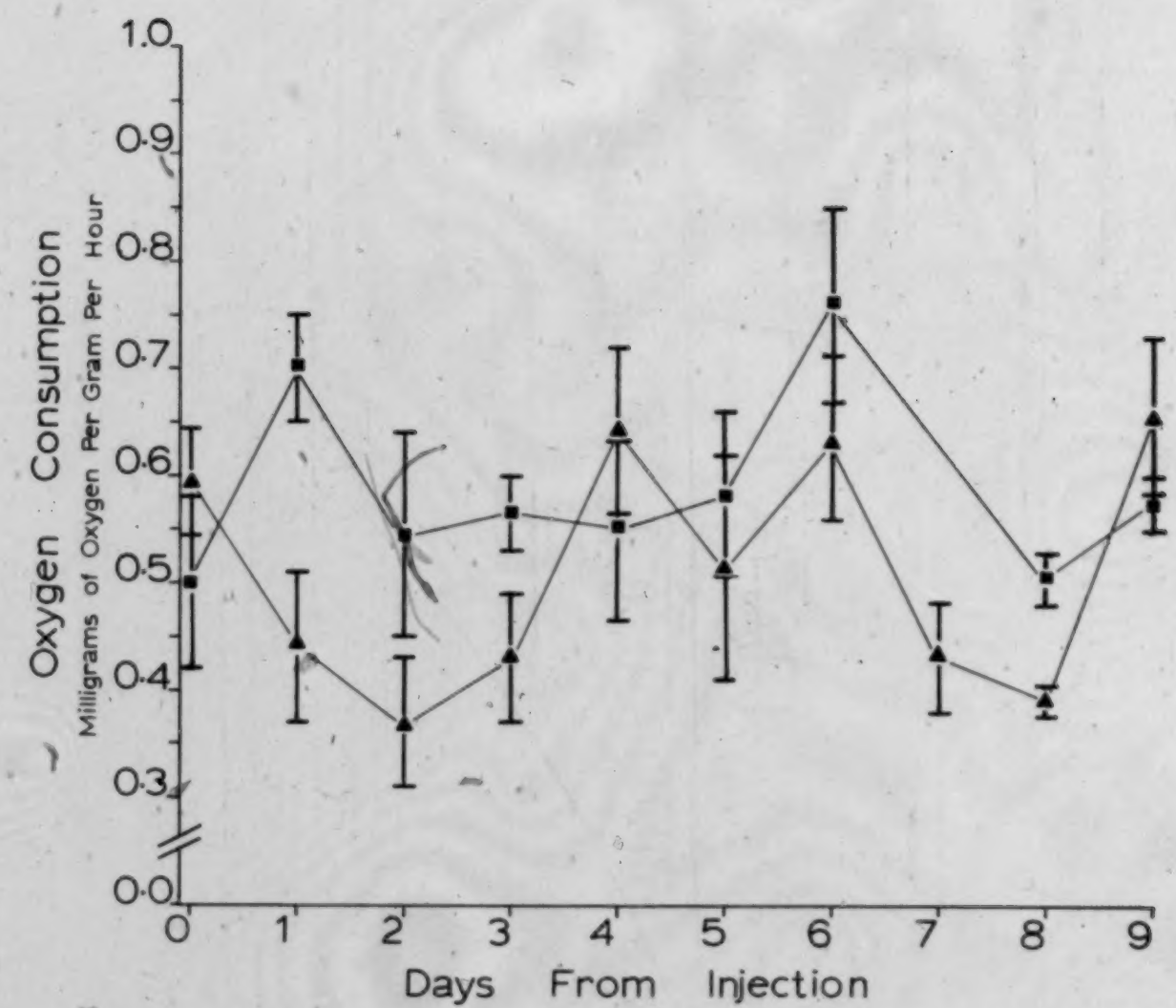

Oxygen Consumption
Milligrams of Oxygen Per Gram Per Hour
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.0
0
1
2
3
4
5
6
7
8
9
Days From Injection

Table 14

WET (LIVE) AND DRY WEIGHTS IN GRAMS OF LARVAL *AMBYSTOMA TIGRINUM*

Wet Weight in Grams	Dry Weight in Grams
69.5	10.2
77.1	9.5
104.0	14.3
71.5	10.3
105.7	18.7
86.2	12.2
116.8	15.7
27.7	4.5
40.6	5.6
90.9	14.7
108.4	15.7
54.2	7.9

Fig. 25. Wet and dry weights of *Ambystoma tigrinum* larvae plotted using a regression equation ($YE = A + (B \times X)$)

$A = -0.14454$
$B = 0.14805$

$$B = \frac{\Sigma XY - \frac{(\Sigma X \times \Sigma Y)}{n}}{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}$$

$$A = \bar{Y} - (B \times \bar{X})$$

▲ - Observed values

● - Points plotted with regression equation

CANADIAN THESES ON MICROFICHE

24001

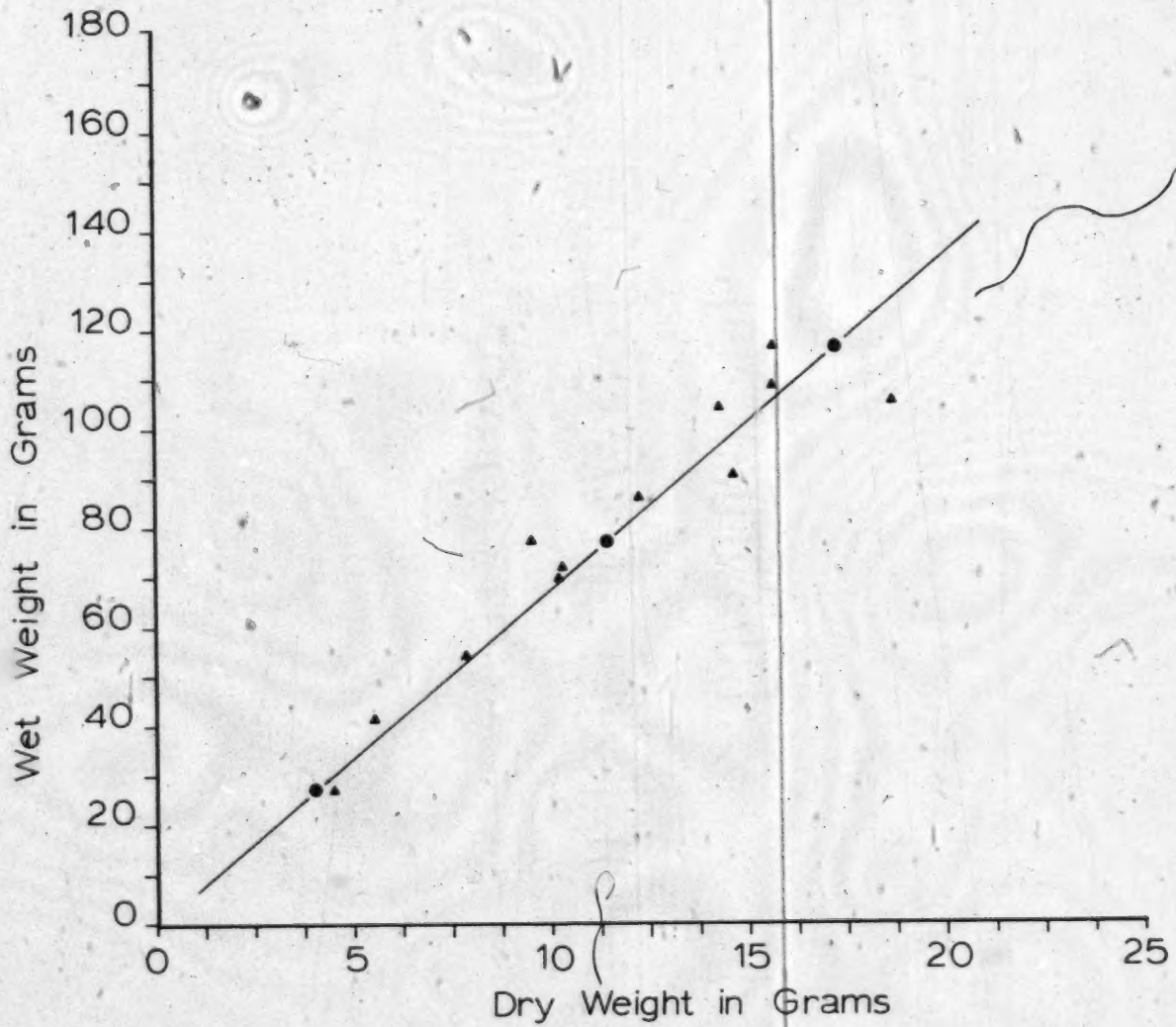
THÈSES CANADIENNES SUR MICROFICHE

AUTHOR/AUTEUR

CORMIE, G.W.

TITLE/TITRE

AN EXAMINATION OF NEOTENY IN AMBYSTOMA TIGRINUM...


180
160
140
120
100
80
60
40
20
0
Wet Weight in Grams
0
5
10
15
20
25
Dry Weight in Grams

DISCUSSION

There are two possible explanations for neoteny in the species of salamander, *Ambystoma tigrinum*, used in this study, the first being an environmental influence and the second hormonal. These are not separate from each other, since environmental influences would eventually have their effects on the hormonal feedback mechanisms that cause the metamorphosis in the salamander.

Temperature is one of the most important environmental influences on salamander metamorphosis (Snyder, 1956; Schrode, 1972). It is suggested that cold inhibits either the release of thyroid hormone or the ability of tissues to respond to its influence. This is borne out by the fact that high altitude lakes in the northern latitudes, where temperatures are low and the summer seasons short, often contain neotenous urodels and anurans alike (Dent, 1968). The American Rocky Mountains often contain neotenous, paedogenetic populations of *Ambystoma tigrinum* (Lynn, 1961). The *Ambystoma tigrinum* used in the present study demonstrate this temperature phenomenon (Table 4, Fig. 23). Animals at colder temperatures required longer periods for metamorphosis and those at 10°C did not undergo thyroxine induced metamorphosis. However, Tyrrell's Lake is not a cold lake as it is maintained by irrigation runoff, snow and rain. The temperature in the lake may rise to as much as 25°C in July and August and the lake is ice free from May to October. Therefore, temperatures would not appear to be a major cause of neoteny in this particular population since a warm period would be available in the summer for metamorphosis.

Light has a slight influence on the growth and metamorphosis of

amphibians. The growth and metamorphosis of *Alytes obstetricans* was shown to be accelerated by supplemental illumination and decreased by increased darkness (Dent, 1968). This phenomenon would appear to have little influence on neoteny in natural populations and has yet to be demonstrated with the exception of cave dwelling amphibians which are often neotenous. Although the *Ambystoma tigrinum* of Tyrrell's Lake appear to be nocturnal and during the daylight hours are bottom dwellers (perhaps burrowing in the mud), their growth does not appear to be adversely affected by this constant low level of light that would occur at the lake bottom and presumably neither would their metamorphosis. On this basis it is assumed that light has no bearing on neoteny of this population of *Ambystoma tigrinum*.

Marzulli (1941) reported that pH of the water influenced the metamorphosing action of thyroxine on *Rana catesbiana* tadpoles. He found that acidity below pH of 4.8 and alkalinity above pH 11 accelerated and retarded, respectively, the metamorphosing action of thyroxine when added to the water. This effect was not observed when the thyroxine was injected into the tadpoles and it was therefore concluded by Marzulli that pH affected the transport of thyroxine across the skin rather than its influence in the animal. The pH of Tyrrell's Lake is 9.1 (Appendix I) and, although this relatively high pH may not affect the action of thyroxine, it may have an influence on its storage and release from the thyroid. This aspect has not been reported on in the literature and may be of some importance in the neoteny of this species. However, the influence of pH on metamorphosis in this species requires further investigation.

Another environmental influence is the presence or absence of chemicals and ions in the aquatic environment that may influence the growth and metamorphosis of amphibians living in it. One of the most prominent substances stressed in the literature is that of iodine and iodine salts (Lynn and Wachowski, 1951). Although iodine is an important constituent of thyroid secretion, its absence in the environment has not yet been reported as a cause of neoteny. In a paper by Dent (1968) it was stated "no one has yet reported finding neotenous larva in water with an iodide content too low to support metamorphosis . . . low iodine content of water is not an ordinary cause of neoteny." Should the absence of sufficient quantities of iodine be a cause of neoteny, then addition of excess iodide in combination with increased temperatures could cause the onset of metamorphosis in *Ambystoma tigrinum*. The results of the iodide experiments in the present study (Table 7) show no indication of this.

Other naturally occurring chemical substances in the water, such as $CaCl_2$, KCl and phosphate ion, were suggested as being possible inhibitors of metamorphosis (Lynn and Wachowski, 1951), but as yet no evidence has been put forward to indicate which substances are involved and what their specific action on metamorphosis is. Tyrrell's Lake has a high level of dissolved solids (5433 mg per liter). However, no attempt was made in the present study to acquire sufficient quantities of sterile filtered lake water to undertake detailed research as to the inhibitory effects, if any, of the ions and chemicals in it. Adult *Rana pipiens* were seen along the shoreline of the lake during the collecting trips, which would seem to indicate that metamorphosis of this species was possible and

that the water of the lake did not contain any such inhibitory substances. It is possible that some combination of ions and pH or chemical runoff from fields surrounding the lake is responsible for a species-specific inhibitory action on the metamorphosis of *Ambystoma tigrinum*, and not on that of *Rana pipiens*.

Crowding and availability of food have been reported as being possible inhibitors of growth and metamorphosis in laboratory situations (Dent, 1968), although there have been no reports of these inhibitory effects occurring in nature. Although the population density of the *Ambystoma tigrinum* larvae appears to be great, there does not seem to be any shortage of *Gammarus sp.*, their principal food in the summer months (Cormie, personal observations).

From observation of the *Ambystoma tigrinum* larva and the results from the thyroxine and TSH experiments, it is possible to suggest a reason for the failure of metamorphosis and the cause of neoteny in this species. Their large size (30 cm or more) would suggest that prolactin is being produced in sufficient amounts from the anterior pituitary to exert its positive influence on growth (Etkin and Gona, 1967; Nicoll, Bern, Dunlop and Strothmon, 1965). In normal tadpoles and larvae this prolactin production tapers off as the influence of the hypothalamus increases, and growth slows (Etkin, 1968). However, no such slowing of growth was seen in these *Ambystoma tigrinum* larvae as animals in length up to 30 cm, that were probably several years old, were frequently found in the lake.

From the thyroxine experiments it is evident that the tissues of the larvae are capable of responding to the thyroid hormone since the

larvae do undergo metamorphosis. This would seem to indicate that lack of tissue response or sensitivity to thyroxine is not the cause of the failure of metamorphosis in this population.

The TSH experiments suggest that the thyroid gland can produce and secrete thyroid hormone in sufficient quantities to promote metamorphosis. Multiple injections seem to be necessary to sufficiently stimulate thyroid hormone production, since a single injection caused only a few of the larvae to undergo metamorphosis. Multiple injections of TSH caused metamorphosis in all of the experimental individuals. Therefore, the inability of the thyroid to produce or secrete thyroid hormones is also not a likely cause of neoteny in this species.

It may be that the failure of metamorphosis arises from a failure in the hypothalamus-pituitary complex, or more specifically the hypothalamus. Since prolactin production by the anterior pituitary is under negative feedback inhibition from the hypothalamus (Etkin, 1968), increased prolactin production in general (and increased growth) would seem to indicate (1) decreased influence of the hypothalamus due to a poorly developed or underdeveloped portal system, or (2) decreased activity of the hypothalamus and subsequent decrease in the amounts of inhibitory factor released. Since growth rates appear to be high (as was stated earlier), it is assumed that prolactin production is high or at least adequate for such a growth rate. Prolactin also has a negative feedback on the production of thyroid hormone (Etkin and Gona, 1967; Gona, 1967; Gona and Etkin, 1970, and Nicoll, Bern, Dunlop and Strothmon, 1965), thus high levels of prolactin would keep the levels of thyroid hormone low and inhibit metamorphosis.

Thyroid hormones are known to stimulate production of release factors from the hypothalamus (Etkin, 1966). From the thyroxine experiments, it is evident that single injections of T_4 initiated metamorphosis in *Ambystoma tigrinum*. It must be assumed, then, that the T_4 caused production of release factors from the hypothalamus (thyrotropic release factor and prolactin inhibitory factor) which in turn increased TSH levels, and decreased prolactin levels. This increase of TSH production and decrease of prolactin production would stimulate the production and release of thyroid hormones and would thereby induce metamorphosis. This complex interaction would not be possible if the hypothalamus-pituitary portal system was not developed sufficiently to transport the release factors and inhibitory factors from the hypothalamus to the anterior pituitary. Likewise it would not be possible if the hypothalamus was not capable of production or release of these factors. On the other hand, because the threshold dose of T_4 was not determined, the amount of T_4 injected may have been sufficient to induce metamorphosis independent of the hypothalamus-pituitary-thyroid complex. The literature seems to favor the former hypothesis.

It seems, therefore, that although the hypothalamus is capable of producing release factors, it does not and, thus, growth is stimulated and metamorphosis is inhibited. The author therefore suggests that some unknown factor(s), acting upon the hypothalamus, inhibits either the production or the release of hypothalamic release factors and thereby causes extended larval life (neoteny) in this population of *Ambystoma tigrinum*.

The artificial staging of induced metamorphosis in neotenic

Ambystoma tigrinum, as set out in the Results, has a number of basic advantages. First, since all the characteristics are externally visible parameters, it is not necessary to kill the animal to determine the stage of metamorphosis. Second, all the selected parameters of stage determination are easily visible with the naked eye, requiring no special equipment such as microscopes and, when taken in combination, easily pinpoint the stage of metamorphosis accurately. As well, the total number of stages is small (six) with only four intermediate stages between the true larva and the adult. Since no complete metamorphic staging information is available in the literature for urodels in general and this species specifically, this system provides a rapid, reliable method of metamorphic staging for any further work with this population of neotenic *Ambystoma*.

Initially it was hoped to measure the oxygen consumption of a large number of *Ambystoma tigrinum* larvae throughout their entire metamorphosis. This did not prove possible due to supply and storage problems with the larvae, as well as fungal infection. Therefore it was not possible to get a sample large enough to conclusively demonstrate a statistically significant overall change of oxygen consumption during the entire metamorphosis of the larvae. It was possible, however, to demonstrate a significant change of oxygen consumption over a shorter period. The oxygen consumption of the larvae was significantly lower at 1 hour and 24 hours post injection of T_4 than in the sham injected controls, thereby demonstrating a depressing effect of T_4 on the metabolism of these *Ambystoma tigrinum* larvae. Since a "normal" non-neotenic population of larva was not available for testing, it is not possible to conclusively

state that this depressing effect of T_4 is a result of the neotenic nature of the population or a species characteristic in general. The decrease in metabolism after injection of T_4 could be due to a cessation of growth in general, thus entering a type of "cellular torpor" prior to metamorphosis or the beginning of cellular catabolism and general absorption of larval tissues, which would not necessarily require extra oxygen (Frieden, 1967, 1968). It has not been conclusively determined in the literature whether amphibians (urodels and anurans) all behave similarly in their metabolic response to thyroxine administration or to metamorphosis (induced or spontaneous). Although many of the cellular and intracellular changes due to T_4 administration have been worked out in detail (Frieden, 1967, 1968; Cohen, 1970), the biochemical aspects have not been assessed in terms of overall tissue demand for oxygen during T_4 administration or spontaneous metamorphosis.

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PLATE 1. Plexiglass chamber used for the measurement of oxygen consumption in the larval *Ambystoma tigrinum*.

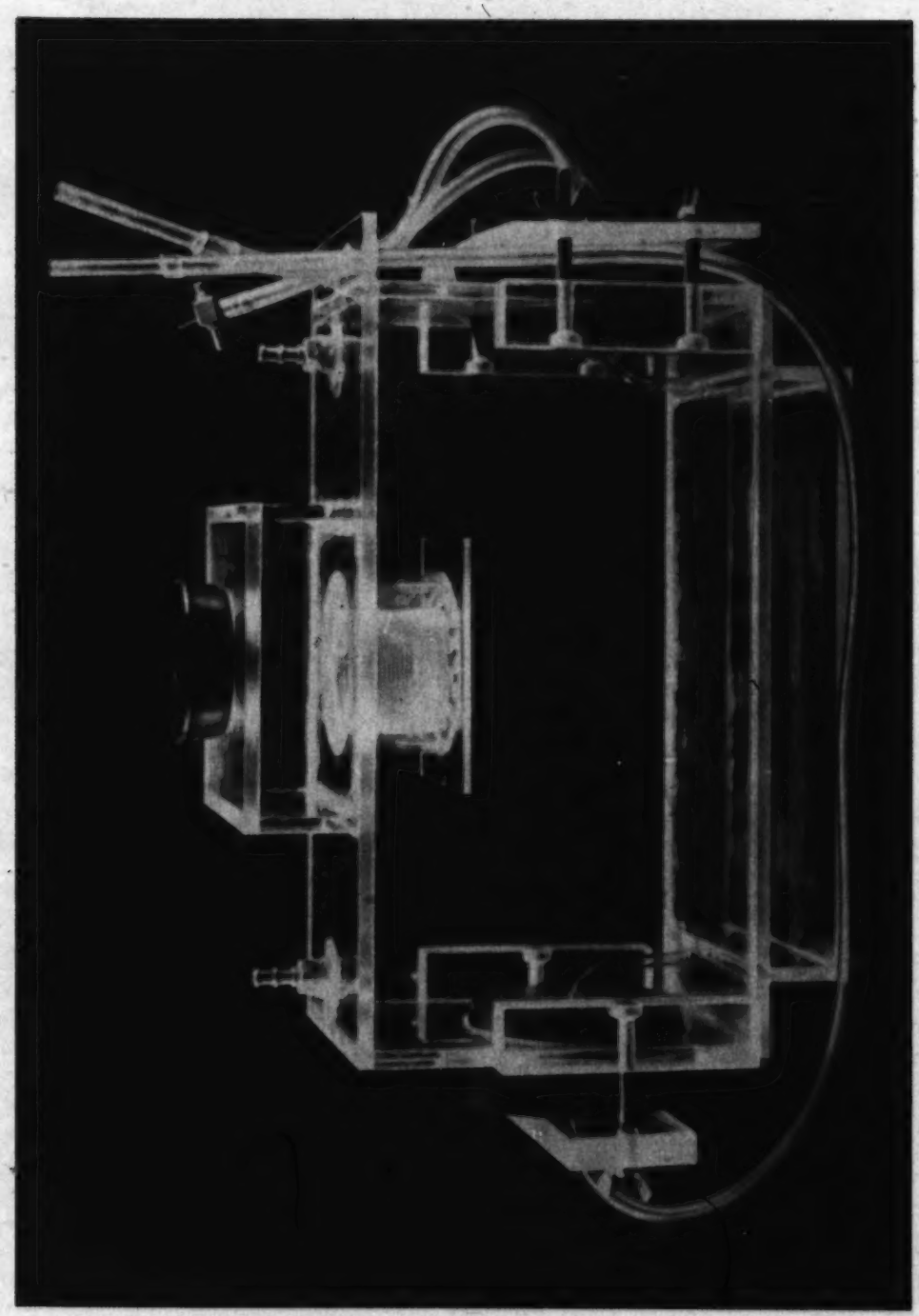
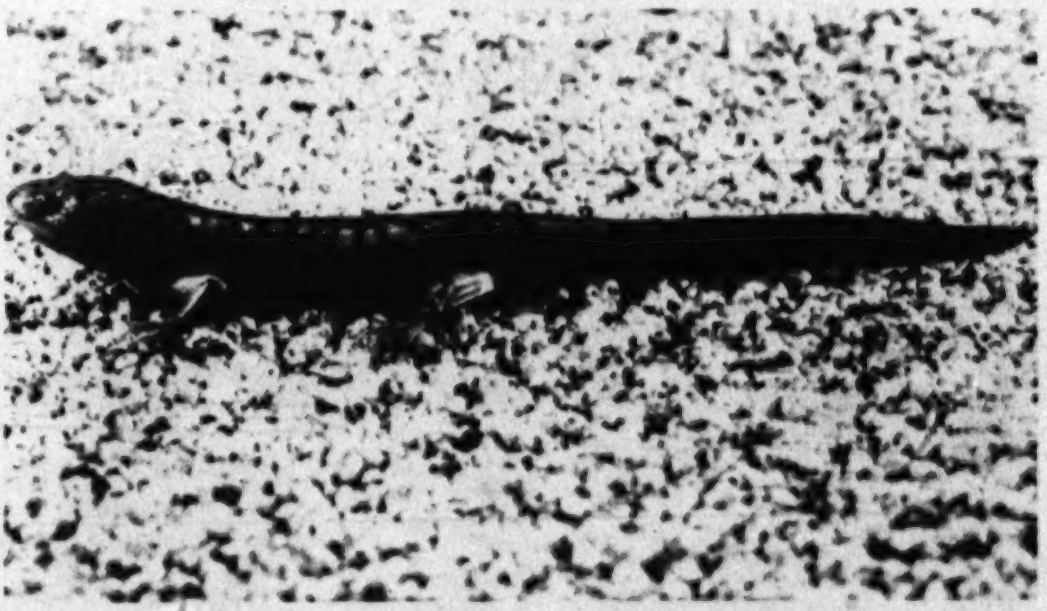


PLATE 2. Frontal view of a larval (Stage I) *Ambystoma tigrinum*.

PLATE 3. Lateral view of a larval (Stage I) *Ambystoma tigrinum*.

PLATE 4. Lateral view of a partially metamorphosed (Stage V) *Ambystoma tigrinum*.

PLATE 5. Lateral view of a partially metamorphosed (Stage V) *Ambystoma tigrinum*.



APPENDIX I

THE METAMORPHIC CHARACTERISTICS OF STAGES I TO VI IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM* LARVA

STAGE I

THE CHARACTERISTICS OF STAGE I IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
labial fold	prominent and well developed
appendages	pectoral - flat and fleshy pelvic - flat and fin-like with a fleshy dorsal lobe
gills	long primary filaments with long, dense blade-like secondary filaments
dorsal tail fin	extends from the posterior of the head region to the tip of the muscular tail, broad base and thin uneven crest, greatest depth above the cloaca
ventral tail fin	extends from the posterior margin of the cloaca to the tip of the muscular tail, rises sharply to the crest in the cloacal region, broad base and uneven crest
gular fold	thin and transparent with a prominent medial slit, not attached to the trunk at the posterior region
digits	broad, flat and fin-like

STAGE II

THE CHARACTERISTICS OF STAGE II IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
labial fold	greatly reduced but present
appendages	both pectoral and pelvic less fleshy and more muscular in appearance with the loss of the dorsal fleshy lobe on the pelvic
gills	reduction in the width and thickness of the primary filaments with corresponding reduction in length of the secondary filaments
dorsal tail fin	absorption and thinning of the anterior portion with thickening and lowering of the posterior crest
ventral tail fin	absorption and thinning of the proximal portion with resultant loss of the crest at the posterior margin of the cloaca
gular fold	loss of transparency, thickening of the posterior margin and loss of the medial slit not attached at posterior margin
digits	loss of blade-like appearance

STAGE III

THE CHARACTERISTICS OF STAGE III IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
labial fold	absent
dorsal tail fin	absorbed posterially to the pelvic region, dorsal to the cloaca; heavily pigmented scar in the region of the absorbed tail fin
ventral tail fin	present only in the distal tail region
gills	primary filaments heavily pigmented, shortened and curled, secondary filaments reduced in number and length but present
gular fold	very thick but not fused to the trunk
eyelids	first evidence of development

STAGE IV

THE CHARACTERISTICS OF STAGE IV IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
dorsal tail fin	absorbed posterially past the cloaca
ventral tail fin	trace amount in the distal tail region
gills	primary filaments reduced to heavily pigmented stubs, secondary filaments absent
eyelids	fully developed
gular fold	center fused to the trunk
gill slits	first two fused shut

STAGE V

THE CHARACTERISTICS OF STAGE V IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
gills	absent, with heavily pigmented gill scars and evidence of unabsorbed hypobranchial apparatus in the region of the pigment
dorsal tail fin	absent, with a heavily pigmented dorsal tail fin scar remaining
ventral tail fin	absent
gular fold	fused to the trunk
gill slits	last gill slit closed
pigmentation	loss of larval grey-green with the acquisition of adult black and yellow

STAGE VI

THE CHARACTERISTICS OF STAGE VI IN THE METAMORPHOSIS OF *AMBYSTOMA TIGRINUM*

Physical Parameters	Characteristics
gill scars	absent
tail fin scars	absent
gular fold	only represented by a smooth crease on the ventral side of the neck region
tail	blunt and muscular

A P P E N D I X II

LAKE SURVEY REPORT CONDUCTED BY A PROVINCIAL FISHERIES BIOLOGIST IN 1962

LAKE SURVEY REPORT

by

R. J. Paterson
(Fishery Biologist)

Name of Lake: Tyrrell's Lake

Location: Sections 17, 18 & 19, Twp. 5, Rge. 17, W 4th
Sections 24, Twp. 5, Rge. 18, W 4th
(Approximately 4 miles SE of New Dayton)

Tyrrell's Lake was visited 17 - 18 July 1960 in company with Fish and Wildlife Officer C. Sawyer. A brief survey was made of some of its physical and biological features. The weather at the time of visit was fine and warm.

General Features -

The lake lies in a shallow coulee in relatively flat prairie country. Its level is maintained by runoff water from the surrounding irrigated land. The lake lies in an approximately NW - SE direction and has an area of approximately 900 acres.

The shoreline is well defined, with a narrow band of weeds extending only a few feet from shore along much of its length. More extensive weed growth was found in the bays at the south end of the lake. However, aquatic vegetation was not found over the larger part of the lake bottom, probably at least partly as a result of the high turbidity.

An interesting feature of the lake was a flock of approximately 200 pelicans. Several species of grebe and duck were also present at the time of visit. Salamanders appeared to be fairly numerous in the lake.

Access -

The lake is surrounded on all sides by private land. Access on this occasion was gained from a road allowance at the South end of the lake. This would be inadequate access for anglers should a sport fishery be developed in the lake. It is understood that there have been negotiations by the County of Warner to lease land between the road and the lake shore at the South end of the lake in exchange for a road allowance on which the landowner has buildings. It is not as yet confirmed that these negotiations have been completed, but if this access is provided then it should be at least adequate for the launching of boats on the lake.

Morphometry -

Several series of soundings were taken by hand line, and from these a contour map of the lake has been constructed. A copy of the map is attached to this report.

Physical and Chemical Features -

Observations on the temperature, dissolved oxygen concentrations and pH were made in 19-20 feet of water, and the results are shown in Table I.

TABLE I. Temperature, Dissolved Oxygen and pH as determined in Tyrrell's Lake 10:30 a.m. 19 July, 1960.

Depth (ft.)	Temperature (°C)	Dissolved Oxygen (cc/l)	pH
0	24.4	8.4	8.2
5	22.7	5.9	
10	21.8	4.8	
15	21.6	2.9	

A heavy bloom of phytoplankton was in progress at the time of visit, and the dissolved oxygen readings indicate a condition of super saturation for approximately the first six feet from the surface. No thermal stratification is apparent but some oxygen depletion is indicated at lower levels during the summer. Owing to the exposed nature of the lake and the winds prevalent in the area it is not expected that any serious oxygen depletion should occur in winter.

The water was fairly turbid at the time of visit. A secchi reading taken with an Ekman dredge indicated 4 1/2 feet. This was taken in the south end in an area where the plankton bloom was less pronounced. The turbidity is probably caused by wave action on the clay soil of the shoreline.

A water sample was taken and analysed by the Provincial Analyst. The total dissolved solids content was 2822 ppm., which is the highest natural concentration we have encountered to date in fishery projects in Alberta. Alkalinity was 455 ppm. which, although well above average, is not exceptional. Sulphate content was 1235 ppm. which is exceptionally high.

Plankton -

As previously stated, a heavy bloom of plankton was in progress at the time of visit. In areas of heavy concentration a coppery-green crust was evident on the surface and filamentous algae were gathering in bundles. Such conglomerations of algae drift on the wind and tend to pile up on the leeward shore. It is unlikely that such a bloom would cause summerkill as dissolved oxygen becomes well distributed into the deeper water and the natural turbidity of the water would restrict the depth of the plankton

bloom to the top 6-7 feet of the lake.

Plankton samples were taken at three locations. Sample I was taken in the south end from a depth of 16 feet. Sample II was taken approximately half way down the lake from 18 feet, and sample III was taken in the north end from a depth of 14 feet. Each of these was a total vertical haul. These samples yielded settled volumes of 21.7 cc., 3.7 cc., and 2.5 cc. respectively. This indicates that the plankton bloom was centered in the shallower areas of the south end of the lake. Examination of these samples and the samples of bottom fauna suggests that the samples were not preserved immediately following collection and that some decomposition had occurred before preservative was added. The large proportion of the material was, as a result, unidentifiable. Specimens of the following species were identified but no attempt is made to estimate abundance:

Phytoplankton

Diatoms:

Fragilaria

Blue Green algae:

Microcystis

Green Algae:

Spirogyra

Pediastrum

Zooplankton

Crustacea:

Diaptomus

Daphnia

It is unfortunate that these specimens were not in good condition as the high salinity of this lake makes its fauna of more than usual interest. It is proposed to collect a further series of samples from this lake at a later date to remedy this situation.

Bottom fauna -

A sample of the bottom fauna was taken using an Ekman dredge in the south end of the lake in 16 feet of water, and in the north end in 12 feet of water. The specimens taken were, however, not identifiable for the above-mentioned reasons.

Fish -

No nets were set in the lake on this occasion. As far as is known there are no fish in this lake. The lake is relatively new in its present form, as it has filled with irrigation runoff since irrigation schemes have been instigated in this area. It has no direct connection with the irrigation ditches.

Conclusions and Recommendations -

This lake has several features which must be considered before it is developed as a sport fishery. It is a relatively large body of water, but angling pressure may be expected to be high. It is therefore essential that good public access is guaranteed before any stocking is attempted.

The lake is subject to high summer temperatures with no distinct thermal stratification, in common with some other lakes in this area. It is also characterised by a very high salinity. We have as yet little experience in Alberta with sport fish in a lake possessing such features.

The author has given some consideration to the possibility of introducing Largemouth Bass to this lake. These fish would probably survive and spawn in the lake, but it is felt that the relatively high turbidity might reduce their success for the sport fishery.

It is proposed that an introduction of rainbow trout fingerlings be

tried in this lake. In view of the size of the lake a large number of fingerlings would be required, in the nature of 250,000 - 300,000. To prevent possible wastage of such a large number of fish and in view of the unusual physical conditions in the lake it would be advisable if some experiments could be carried out to determine whether such conditions could have an adverse effect on survival. Live cages could be suspended in the lake for 2-3 months in midsummer, to test the survival of hatchery trout. A comparable lake in the area, possibly Ross Lake, could be used as a control. Other experiments, such as a closed circuit hatchery trough employing water from the lake could be used to test the effects of the salinity only and exclude the effects of temperature.

It is proposed that largemouth bass should be considered as an alternative species if the sport fishery for trout should not prove successful. This species would have the advantage of continued natural reproduction, whereas no trout reproduction can occur in the lake and reintroduction of trout cannot be carried out until the first population of introduced trout has been reduced to the point where it will not significantly interfere with a second introduced population. It would however be preferable if a lake more suited to this species could be used first for an experimental introduction of the species to determine its suitability for the sport fishery and its potential use in southern Alberta.

As a precautionary measure, it is proposed that a standard gang of nets be set in the lake to check for any fish which may have been accidentally or deliberately introduced to the lake.

WATER ANALYSIS REPORT

Lake TYRRELL LAKE Analysed by Provincial Analyst

Date July 26, 1960.

Total solids	2522
Ignition loss	308
Hardness	400
Sulphates	1235
Chlorides	44
Alkalinity	455
Nature of alkalinity	Bicarbonate of lime and magnesium.
Nitrites	Nil
Nitrates	Nil
Iron	1.8
Fluorine	
Other (specify)	

Remarks:-

Water chemically unsuitable due to high Glauber's salt content 153.0 grains/gallon. This makes water laxative.

APPENDIX III

WATER ANALYSIS OF TYRRELL'S LAKE CONDUCTED BY THE DEPARTMENT OF ZOOLOGY WATER ANALYSIS LABORATORY

Water Analysis of Tyrrell Lake

Date Sampled: June 1973

Depth: Shoreline

Sampling Location: Collecting Site(see Materials and Methods)

Alkalinity, Phenl. as $CaCO_3$	76.5	mg./l.
Alkalinity, Total as $CaCO_3$	636.2	mg./l.
Color	47	
Hardness, Ca as $CaCO_3$	146	mg./l.
Hardness, Total as $CaCO_3$	872	mg./l.
Iron	0.24	mg./l.
Nitrate Nitrogen		mg./l.
pH	9.10	
Phosphate, Ortho	0.29	mg./l.
Silica	1.16	mg./l.
Specific Conductance	8000	
Sulfate	3700	mg./l.
Total Dissolved Solids	5433	mg./l.
Turbidity	12	
Chloride	156.6	

APPENDIX IV

THE MORTALITY IN SHAM AND L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM* LARVA AT 10, 15, 20 AND 25 DEGREES CENTIGRADE OVER A PERIOD OF 46 DAYS

Temperature in Degrees Centigrade

Days from injection	10		15		20		25	
	T_4	Sham	T_4	Sham	T_4	Sham	T_4	Sham
1	0	0	0	0	1	0	0	0
2	0	0	0	0	0	0	0	1
3	0	0	0	0	0	1	0	0
4	0	0	0	0	0	2	0	0
5	1	1	0	0	1	0	0	0
6	0	1	0	0	1	0	1	0
7	0	0	2	0	0	0	2	0
8	0	0	0	1	2	0	1	0
9	0	0	0	2	1	0	1	0
10	0	0	2	0	1	0	1	0
11	0	0	0	0	1	0	2	0
12	0	0	0	0	2	0	0	0
13	0	0	0	0	0	1	0	0
14	0	0	0	1	0	0	0	0
15	0	0	0	0	0	0	0	1
16	0	0	0	0	0	0	0	2
17	0	0	0	1	0	0	0	0
18	0	0	0	0	0	1	0	0
19	0	0	0	0	0	1	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	1	0
24	0	0	0	0	0	0	0	1
25	0	0	1	0	0	0	0	1
26	0	0	1	0	0	1	0	0
27	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0
30	3	0	0	0	1	0	0	0
31	0	0	2	0	0	0	0	0
32	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0
39								
40-46								

APPENDIX V

DRY WEIGHTS IN SHAM AND L-THYROXINE INJECTED *AMBYSTOMA TIGRINUM*

Dry Weight in Grams*

Sham Injected	L-thyroxine Injected
16.881	19.102
17.251	17.029
11.033	14.142
13.994	15.253
17.547	19.546
15.475	15.697
15.179	12.440
16.141	13.328
16.776	12.588
15.253	15.919
15.874	14.216
13.891	13.328
17.325	13.920
15.696	11.033
15.919	13.076
13.180	14.764
15.475	15.549
13.624	15.031
16.363	10.488
15.889	11.130
21.397	22.359
16.955	19.176
12.292	17.177
15.549	18.732
19.176	19.102
17.251	20.138
16.881	16.807
15.475	12.366
16.585	17.325
17.770	16.659
16.659	15.031
15.327	12.588
16.348	16.437
16.570	11.329
13.920	16.437
16.955	15.549
15.475	15.771
10.663	14.586
13.402	12.567
16.393	13.210
$\bar{X}$ ± S.E.M.	$\bar{X}$ ± S.E.M.
15.695 ± 0.312	15.273 ± 0.434

*Determined from regression equation.

APPENDIX VI

APL COMPUTER PROGRAM FOR THE DETERMINATION OF OXYGEN CONSUMPTION OF *AMBYSTOMA TIGRINUM* LARVA

```
     ∇OXY[⎕]∇
     ∇ OXY N;ANS1;ANS2;BFOR;AFTR;M;EM;ST;MG;ML;MGOO;Z;TMG;TML;TIM;A;B;C;D;N;I;R;RESPMG;RESPML;TOMG;TOML
[1]    'NUMBER OF ANIMALS
       '
[2]    N←⎕
[3]    'ENTER MATRIX ST'
[4]    A←⎕
[5]    B←⎕
[6]    ST←(N,2)⍴A,B
[7]    'ENTER MATRIX EM'
[8]    C←⎕
[9]    D←⎕
[10]   EM←(N,3)⍴C,D
[11]   I←1
[12]   R←(N,5)⍴1
[13] LOOP:ANS1←(+/(ST[I;]*2))*(⍴ST[I;])
[14]   ANS2←(+/(EM[I;]*2))*(⍴EM[I;])
[15]   BFOR←ANS1×21.27×M[I;6]
[16]   AFTR←ANS2×21.27×M[I;6]
[17]   MG←((M[I;7]÷1000)×BFOR)-((M[I;7]÷1000)×AFTR)
[18]   ML←((BFOR÷1.43)×(M[I;7]÷1000))-((AFTR÷1.43)×(M[I;7]÷1000))
[19]   Z←((M[I;4]÷760)×(M[I;8]÷1000))÷(0.08210000000000001×(M[I;10]+273.15))
[20]   MGOO←Z×(2×15.99491)×1000
[21]   TMG←MGOO+MG
[22]   TML←M[I;8]+ML
[23]   TIM←M[I;8]×60
[24]   TOMG←TMG÷TIM
[25]   TOML←TML÷TIM
[26]   RESPMG←TOMG÷M[I;5]
[27]   RESPML←TOML÷M[I;5]
[28]   R[I;]←M[I;3],TOMG,TOML,RESPMG,RESPML
[29]   I←I+1
[30]   →(I≤N)/LOOP
[31]   ⍉0
[32]   ⍉0
[33]   ⍉0
[34]   'DATE:  ';M[1;1]
[35]   'SECTION NUMBER:  ';M[1;2]
[36]   ⍉0
[37]   ⍉0
[38]   20⍴' ';'OXYGEN CONSUMPTION DATA'
       '
[39]   'ANIMAL NUMBER ';2⍴' ';'MG./HR. ';6⍴' ';'ML./HR. ';4⍴' ';'MG./GM./HR. ';4⍴' ';'ML./GM./HR.'
       '
[40]   75⍴'-'
[41]   7 0 15 4 13 4 13 4 15 4 DFT R
     ∇
```